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The Dynamics of Protein Metabolism. I. The Interrelationship Between Protein and Caloric Intakes and Their Influence Upon the Utilization of Ingested Protein for Tissue Synthesis by the Adult Protein-Depleted Rat. Earl P. Benditt, M.D., Eleanor M. Humphreys, M.D., Robert W. Wissler, Ph.D., C. Harold Steffee, M.A., Laurence E. Frazier, B.A., and Paul R. Cannon, M.D., Chicago, Ill. ---- 257

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THE DYNAMICS OF PROTEIN METABOLISM

I. The Interrelationship Between Protein and Caloric Intakes and Their Influence Upon the Utilization of Ingested Protein for Tissue Synthesis by the Adult Protein-Depleted Rat

Earl P. Benditt, M.D., Eleanor M. Humphreys, M.D., Robert W. Wissler, Ph.D., C. Harold Steffee, M.A., Laurence E. Frazier, B.A., and Paul R. Cannon, M.D.
Chicago, Ill.

INTRODUCTION

OPTIMAL nutrition remains a major problem of medicine and surgery despite the accumulating knowledge concerning vitamins, amino acids, and other essential dietary factors. Until certain relationships are more clearly elucidated, however, we still cannot utilize this knowledge with full effectiveness. Important among these is the interrelationship between the level of protein intake, the energy intake, and the utilization of dietary protein. The present studies were undertaken in order to examine this interrelationship under controlled conditions. The basic plan was to study the rate of utilization of protein for tissue fabrication by the protein-depleted animal under the following circumstances: (1) varying caloric intake with constant protein intake; (2) varying protein intake with constant caloric intake; and (3) simultaneous variation of caloric and protein intakes.

In these studies we have examined not only gross rates of fabrication of protein in the body as a whole, but also rates in various body compartments. This paper deals only with the gross metabolism of protein in the rat. A second paper will contain evidence which demonstrates a fundamental similarity of the protein fabricating mechanisms of the rat and man. Later papers will deal with the relative rates of protein synthesis in various body compartments of protein-depleted animals.

MATERIALS AND METHODS

In these experiments protein-depleted, young adult male albino rats (Sprague-Dawley) were used. At the start of the depletion period their weights averaged 210 grams. For two and one-half months they were depleted upon a low protein diet which contained adequate offerings of calories, vitamins, minerals, and other dietary essentials. The depletion diets and regime have been described.[1] At the end of the depletion period the average weight of the animals was 154 grams. Animals were then individualized. Groups of five or six animals

From the Department of Pathology, University of Chicago.

The subject matter of this paper has been undertaken in cooperation with the Navy Department Office of Naval Research and with the Committee on Food Research of the Quartermaster Food and Container Institute for the armed forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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were selected to be fed each of the experimental diets. The animals were chosen so that each group was comparable with respect to initial weight, weight loss, serum protein concentration, and hemoglobin concentration.

The basic composition of the diets was the same in all three experiments. For the protein, a half-and-half mixture of bovine lactalbumin* and casein† vitamin test was used. The composition of the diets is given in Tables I and II. It is evident from Table I that in Experiment 1 the absolute quantity of protein offered per day was constant, but the quantity of carbohydrate varied. In Experiment 2 the absolute quantity of protein fed per day was varied and the total caloric value of the diet offered was maintained constant. Experiment 3 utilized the same diet throughout, offered in different amounts to different groups of animals. Diets were offered in weighed amounts each day. Diet consumption was measured daily and the experimental feeding period was fourteen days.

TABLE I. COMPOSITION OF DIETS

DIET	DIET FED PER DAY (GM.)	PROTEIN OFFERED PER RAT PER DAY (GM.)	CALORIES OFFERED PER RAT PER DAY	INGREDIENTS PER 100 GM. OF DIET (GM.)							
				FIBER	CORN OIL	LACTALBUMIN	CASEIN	DEXTRIN	SALT MIX	LIVER CONCENTRATE	WATER
Experiment 1											
A	4.8	1.56	15	4.9	11.3	18.1	16.3	20.3	12.5	3.1	6.4
B	8.0	1.56	25	4.9	6.8	10.8	9.8	43.5	7.5	1.9	10.6
C	10.5	1.56	35	5.2	5.1	8.3	7.5	56.9	5.7	1.4	6.7
D	15.0	1.56	48	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6
E	18.0	1.56	60	5.2	3.0	4.8	4.4	67.9	3.3	0.8	8.6
F	15.0	----	48	5.0	4.0	0.0	0.0	71.0	4.0	1.0	12.7
Experiment 2											
A	15.0	0.53	48	5.0	3.9	1.8	1.6	68.0	4.0	1.0	12.4
B	15.0	0.83	48	5.0	3.8	3.0	2.7	66.0	4.0	1.0	12.2
C	15.0	1.14	48	5.0	3.7	4.3	3.8	64.0	4.0	1.0	11.9
D	15.0	1.51	48	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6
E	15.0	1.79	48	5.0	3.5	7.3	6.5	59.1	4.0	1.0	11.4
F	15.0	2.87	48	5.0	3.1	11.6	10.5	51.9	4.0	1.0	10.6
Experiment 3											
A	4.7	0.46	15	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6
B	7.8	0.76	25	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6
C	10.9	1.06	35	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6
D	15.0	1.46	48	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6
E	18.7	1.82	60	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6
F	4.7	0.48	15	5.0	3.6	5.8	5.2	61.5	4.0	1.0	11.6

Initial determinations of the various blood compartments were made by methods which have been described in detail elsewhere.[2] At the end of the test period these values were redetermined. The animals were then sacrificed. Liver, heart, kidneys, and approximately 40 per cent of the blood were removed. The gastrointestinal tract was stripped of its contents. The carcass with the residual contents, including the gastrointestinal tract, was weighed and analyzed for water, fat, protein, and ash.

*Borden's No. 1542.
†SMACO, vitamin test, General Biochemicals Inc.

TABLE II. DAILY VITAMIN RATION*

VITAMIN	QUANTITY OFFERED PER DAY
Thiamin chloride	80 gamma
Riboflavin	120 gamma
Niacin	200 gamma
Pyridoxine HCl	90 gamma
Calcium pantothenate	190 gamma
Choline chloride	27 mg.
Oleum percomorphum	
Vitamin A	60 U.S.P. units
Vitamin D	9 U.S.P. units

*All diets offered these quantities of the vitamins per animal per day except in Experiment 3. In this experiment the vitamin offering was proportional to the quantity of diet offered, diet 3-D providing the standard allowance. Diet 3-F, otherwise identical with 3-A, offered the standard quantities of the vitamins.

The method of carcass analysis was as follows. Each carcass was cut into pieces with a scissors and the pieces ground in a motor-driven meat grinder. Grinder and parts were rinsed with alcohol and wiped with a weighed filter paper. Grindings, washings, and paper were collected in a weighed evaporating dish and dried at a temperature of 60° C. in a hot-air oven for a minimum of five days. Following this, the dish and contents were weighed and the contents were transferred to a weighed fat-free muslin sack and extracted for twelve hours with alcohol in a Soxhlet extractor. They were re-extracted for twelve hours with ether. Following this, the sack and contents were dried again for forty-eight hours in the 60° C. oven, cooled in a desiccator, and weighed. Preliminary to ashing, the dried fat-free carcass was ground to a fine powder in a motor-driven pulverizer. Dry 2 Gm. samples were ashed in a muffle furnace at 600° C. for six hours, cooled in a desiccator, and weighed.

It was found in early experiments that complete recovery of the lost weight by these protein-depleted adult animals was not associated with significant changes in the ash content of the carcass and that differences of the order of magnitude found were within the limits of variation among individual animals. Therefore, in Experiments 2 and 3 the ash content was determined on single samples from each of the diet groups and the pooled average so obtained was used to compute the protein content of each carcass.

Protein was computed by deducting the weight of the ash from that of the dry fat-free carcass. In order to ascertain how closely this value approximated the conventional $N_2 \times 6.25$ value for the estimation of the protein the following experiment was performed. Nitrogen determinations were made on seven carcasses of animals of widely differing weights. The nitrogen concentration of the ash-free carcass was found to be 15.7 ± 0.03 per cent. This value is in reasonable agreement with the commonly accepted value for animal proteins of 16.0 per cent nitrogen and with the value of 15.4 per cent found by Addis and co-workers.[3]

The livers were analyzed for fat, water, protein, and water-soluble constituents by a method similar to that used for the carcasses. The details will be given in a later paper. All hearts and kidneys were weighed and selected ones were analyzed.

OBSERVATIONS

Table III presents the observations on body weight, diet consumption, carcass fat and protein. Recorded in Table IV are the averages of the mean weights of the animals for the fourteen-day experimental period, the average weight gains, the calculated surface areas,[4] the average daily protein intakes, caloric intakes, and rates of protein gain.

The protein intakes are recorded as grams of protein ingested ($N_2 \times 6.25$) per kilogram of body weight per day. The rate of protein utilization is recorded in an analogous fashion as grams of protein gained per kilogram of body weight per day. Caloric intake appears as calories per square meter of body surface per day. These units may appear unnecessarily devious and cumber-

some; an inspection of the nature of the quantities, however, shows that they reduce the measured values to a system of units which is independent of the size of the animal. The advantages of this for comparing animals of different sizes within the same species or of different species are obvious and will become clearer.

TABLE III. ORIGINAL DATA FOR BODY WEIGHTS, DIET CONSUMPTION, CARCASS FAT AND PROTEIN*

DIET	NUMBER OF ANIMALS	PREDEPLETED WEIGHT (GM.)	DEPLETED WEIGHT (GM.)	WEIGHT AFTER 14 DAYS REPLETION (GM.)	DIET CONSUMED IN 14 DAYS (GM.)	CARCASS FAT (GM.)	CARCASS PROTEIN (GM.)
				Experiment 1			
A	4	211 ± 1.7	148 ± 2.5	158 ± 2.0	67 ± 0.2	11.0 ± 1.55	27.9 ± 0.96
B	5	216 ± 3.6	153 ± 2.3	181 ± 1.8	112 ± 0.1	12.0 ± 1.62	32.6 ± 0.24
C	5	211 ± 2.6	151 ± 3.2	200 ± 3.0	146 ± 0.4	25.0 ± 1.52	35.7 ± 0.71
D	5	212 ± 4.7	153 ± 3.1	207 ± 2.0	206 ± 0.9	33.1 ± 1.93	34.8 ± 0.26
E	5	210 ± 1.5	150 ± 2.7	215 ± 2.9	233 ± 4.0	36.4 ± 1.23	35.2 ± 0.73
F	4	214 ± 3.0	158 ± 3.9	146 ± 1.5	119 ± 1.7	11.4 ± 0.67	25.9 ± 0.36
				Experiment 2			
A	5	205 ± 1.8	146 ± 2.8	156 ± 2.3	165 ± 5.5	19.2 ± 1.09	26.7 ± 0.44
B	6	208 ± 2.6	146 ± 1.5	188 ± 1.3	201 ± 1.5	27.8 ± 0.97	32.1 ± 0.55
C	6	208 ± 2.7	147 ± 2.1	208 ± 2.6	207 ± 0.1	29.0 ± 1.48	35.5 ± 0.38
D	6	206 ± 1.4	148 ± 1.8	222 ± 1.5	206 ± 0.8	30.9 ± 2.63	35.9 ± 0.56
E	6	207 ± 2.9	145 ± 2.7	229 ± 3.5	204 ± 0.7	30.1 ± 2.36	38.2 ± 0.91
F	6	207 ± 3.7	145 ± 3.4	235 ± 3.4	206 ± 0.2	26.1 ± 0.50	40.3 ± 0.70
G	5	208 ± 3.2	149 ± 3.8	132 ± 3.8	129 ± 3.9	10.5 ± 1.16	25.2 ± 0.66
				Experiment 3			
A	4	214 ± 1.9	155 ± 2.5	159 ± 2.2	66 ± 0.0	13.6 ± 0.32	29.6 ± 0.74
B	5	214 ± 3.5	154 ± 2.1	181 ± 2.1	109 ± 0.2	20.2 ± 1.07	32.3 ± 0.31
C	5	213 ± 1.8	158 ± 2.8	204 ± 3.8	151 ± 0.3	25.2 ± 2.36	35.9 ± 1.00
D	5	214 ± 2.2	157 ± 3.4	229 ± 3.3	208 ± 0.6	25.1 ± 2.26	38.0 ± 0.75
E	5	215 ± 3.8	153 ± 4.5	240 ± 2.3	247 ± 3.2	40.7 ± 1.95	38.7 ± 0.55
F	5	215 ± 3.0	156 ± 3.6	158 ± 3.1	66 ± 0.2	9.0 ± 1.11	30.5 ± 0.68
G	5	213 ± 3.4	155 ± 1.9	135 ± 1.7	111 ± 4.2	13.2 ± 1.01	25.2 ± 0.42

*The values given are means and standard errors for animals fed each diet. Standard errors were computed by: S.E. = $\sqrt{\frac{\Sigma (x-m)^2}{n(n-1)}}$

Protein gains were measured from an initial value for carcass protein estimated in the following manner. In each experiment a control group of depleted animals was maintained on the low protein ration through the experimental period. This group was sacrificed with the others. The mean quantity of protein found in the carcasses of these animals was subtracted from the quantity determined in each of the repletion groups. The value thus obtained included the quantity of protein lost by the control group, that is, the wear and tear protein loss. This quantity we have determined and subtracted from the apparent gains of the animals in the repletion groups in order to obtain the measure of protein fabrication over and above that which merely replaced the wear and tear losses.

In order to include all, or practically all, of the protein fabricated in the experimental interval, the increase in the liver protein was added to that in the carcass protein. Increases in other compartments not included in the car-

TABLE IV. WEIGHT GAINS AND RATIO OF PROTEIN UTILIZATION AT VARIOUS CALORIC AND PROTEIN INTAKE LEVELS

NUMBER OF ANIMALS	AVERAGE MEAN WEIGHT FOR 14 DAYS* (GM.)	AVERAGE WEIGHT GAIN FOR 14 DAYS (GM.)	SURFACE AREA AVERAGE FOR 14 DAYS (M^2)	DAILY PROTEIN INTAKE (GM./KG.)	DAILY CALORIC INTAKE (CAL./M^2)	DAILY PROTEIN GAIN (GM./KG.)
			Experiment 1			
4	159	6	.026	9.6	560	0.3
5	168	34	.027	8.9	900	2.3
5	177	61	.028	8.6	1240	3.5
5	182	67	.028	8.3	1670	3.1
5	187	76	.029	7.3	1840	3.2
4	153	−18	.026	0.4	1060	—
			Experiment 2			
5	154	13	.026	2.8	1510	0.0
6	168	41	.027	4.8	1690	2.6
6	178	61	.028	6.3	1690	3.7
6	186	74	.029	8.0	1640	3.7
6	188	85	.029	9.3	1610	4.6
6	191	90	.029	14.8	1610	5.3
5	140	−71	.024	0.5	1210	—
			Experiment 3			
5	157†	2	.026	3.4	570	1.8
4	154	3	.026	2.9	580	1.4
5	162	28	.027	4.6	930	2.5
5	179	50	.028	5.9	1220	3.8
5	193	72	.030	7.5	1610	4.4
5	197	87	.030	8.7	1885	4.5
5	145	−20	.025	0.4	1040	—

*Mean weight is the average of the initial and final weights of the animals in each group for the fourteen-day period.

†High vitamin diet.

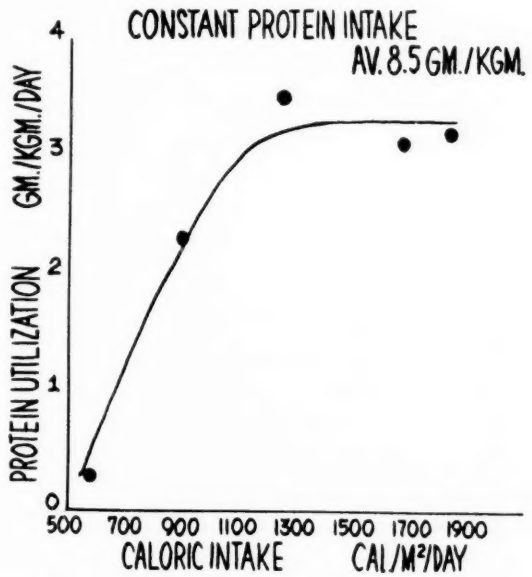

Fig. 1.—The influence of level of caloric intake on protein utilization.

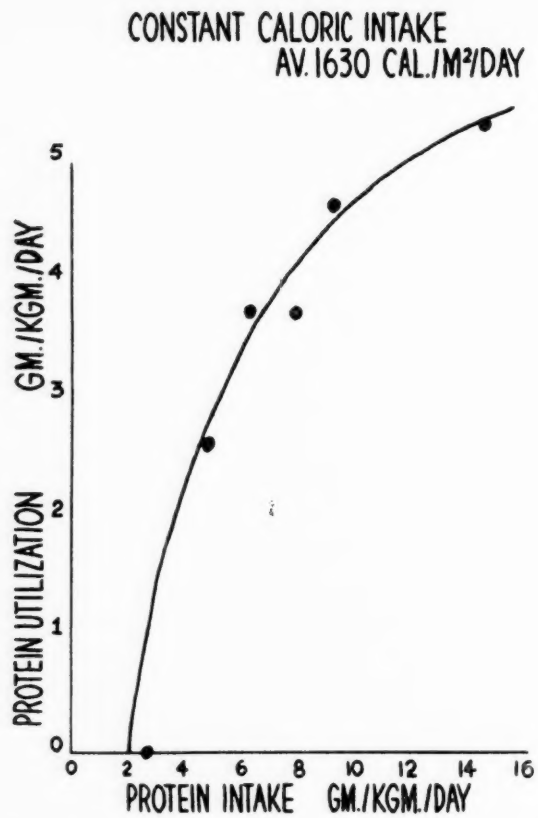

Fig. 2.—The influence of level of protein intake on protein utilization.

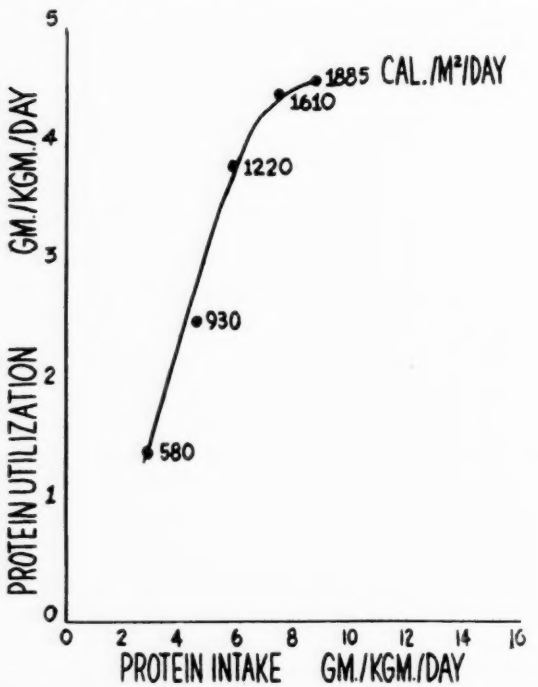

Fig. 3.—The influence of simultaneous variation of protein and caloric intake levels on protein utilization.

cass were not of sufficient magnitude to necessitate their addition. Recorded rates of gain therefore represent, for practical purposes, all of the protein fabricated into body tissue, exclusive of that used for maintenance.

Fig. 1 demonstrates the influence of variation in caloric intake upon protein utilization, the protein intake remaining approximately constant. At the lowest level of caloric intake (560 Cal. per square meter per day) protein utilization was very poor (0.3 Gm. per kilogram per day). With increasing caloric intake, protein utilization rose until a maximum was reached at an intake level of 1,240 Cal. per square meter per day. Beyond this point there was no additional utilization of protein despite the fact that the caloric intake increased up to 1,840 Cal. per square meter per day.

The effect of varying the protein intake at a constant caloric intake is demonstrated by Fig. 2. As the protein intake was raised from 2.8 to 14.8 Gm. per kilogram per day, protein utilization rose. The rise was almost linear up to about 6 Gm. per kilogram per day; beyond this point the rate of protein utilization became progressively less. Nevertheless, at the highest level of intake the ceiling of utilization had not yet been reached.

What occurred when increasing quantities of the same diet were fed to the depleted animals is shown in Fig. 3. In this situation there is a constant ratio of protein to energy in the diet, the levels of protein and caloric intake varying

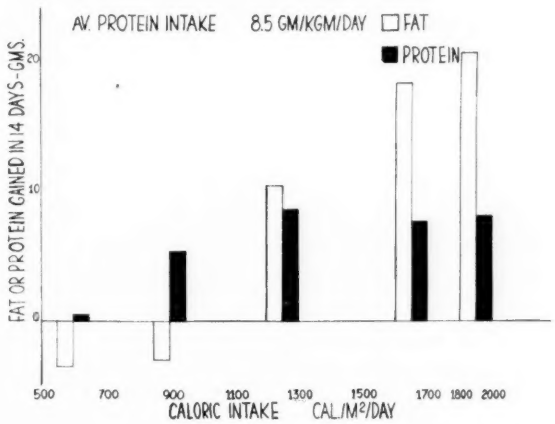

Fig. 4.—The influence of caloric intake at a constant protein intake on the rate of fabrication of carcass fat and protein by depleted rats.

together. Under these circumstances the rate of protein utilization increased with increasing diet intake. Looked at from another point of view, when the quantity of diet was restricted, the rate of protein utilization was restricted. It is evident from the data that the limitation in the rate of protein utilization was not the summation of the effects of the restriction of both protein and energy intakes, and the restriction of utilization at any point was no greater than the maximum restriction induced by the restriction of the protein component alone.

The quantity of fat deposited at a constant protein intake and increasing caloric intakes was approximately proportional to the caloric intake over the range studied (Fig. 4). Comparison of the crude weight gains (Table III, Experiment 1) with the measured fat gains (Fig. 4) demonstrates that the body weight gains at caloric intake levels above 1,240 Cal. per square meter per day were largely due to the increase in carcass fat. On the other hand, when the caloric intake was maintained constant and the protein intake varied (Fig. 5), fat was gained in appreciable quantities at all levels of protein intake above the zero level. The fat gains increased with increasing protein intake up to levels of 8 Gm. per kilogram per day. Further increase in the protein intake resulted in moderate decreases in the quantity of fat deposited since the protein calories were increased at the expense of the nonprotein calories of the diet.

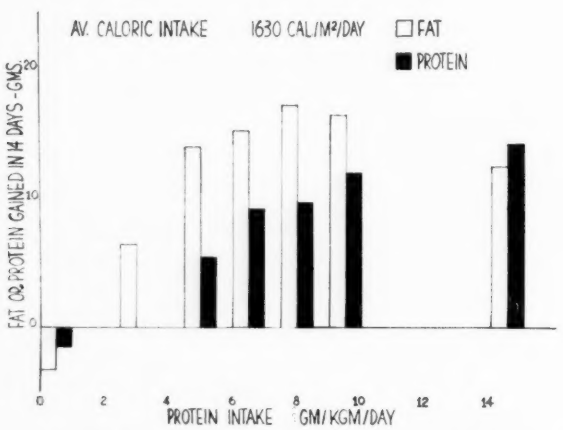

Fig. 5.—The influence of protein intake at a constant caloric intake on the fabrication of carcass fat and protein by depleted rats.

DISCUSSION

In 1919 Osborne, Mendel, and Ferry[5] stated that, "Economy in nutrition during growth depends upon the correct adjustment between the proportions of protein and total energy supplied. . . ." It seems reasonable that the same should hold for the reconstruction of lost tissue. If so, we are faced with the problem of finding the optimal proportions of these factors for tissue synthesis. In order to arrive at these values it is necessary to examine the more general interrelationship between the energy and protein components of the diet and their mode of influence upon protein utilization.

It is recognized that for the optimal storage of nitrogen during growth an "adequate" caloric intake must be maintained.[6] In the convalescent patient a sufficient quantity of energy-containing nutriment must be supplied along with administered amino acids in order for nitrogen retention to occur.[7] In growing rats on a constant protein intake, the restriction of the caloric intake below a critical level results in a diminished rate of nitrogen storage. Moreover, increasing the caloric intake above this level does not effect an increase in the utilization of a given quantity of protein.[8]

Our observations on protein-depleted animals are in agreement with those cited on patients and growing animals. Thus there is a restriction in the utilization of a constant quantity of protein when the caloric intake falls below a critical value of about 1,240 Cal. per square meter per day. Furthermore, elevation of the caloric intake above this level does not increase the effectiveness of the dietary protein for the synthesis of new tissue. It is of interest in this connection that the critical caloric value observed by Bosshardt and co-workers[8] with the *growing* rat was approximately 1,250 Cal. per square meter per day.

What is the significance of this critical caloric value? It is possible to elucidate the nature of the composition of this 1,240 Cal. per square meter per day by breaking it down into its component parts: (a) energy used for maintenance; (b) energy for physical work; (c) energy for tissue fabrication; (d) energy stored in the form of new tissue (that is, as fat and protein); and (e) waste energy in compounds excreted but not completely burned. From carcass analysis data and food intake energy balances can be constructed. Thus the animals receiving a total energy allowance of 1,240 Cal. per square meter per day stored 323 Cal. per square meter per day as new tissue. The difference between the total intake and the energy appearing as new tissue represents the quantity consumed for energy and wasted. Therefore the animals must have consumed and wasted a total of 917 Cal. per square meter per day. From Table III it is evident that the animals which received 560 Cal. per square meter per day were able just to maintain themselves during the fourteen-day period. It appears justifiable therefore to take this as an approximation to the minimal maintenance energy requirements. The difference between the total energy consumed (917 Cal. per square meter per day) and the energy used for maintenance (560 Cal. per square meter per day) represents the energy used to construct new tissue plus waste energy, in this case 457 Cal. per square meter per day.

The quantity 1,240 Cal. per square meter per day therefore appears to represent the energy allowance which will supply the maintenance needs of the animal with sufficient excess to allow for maximal utilization of ingested protein for tissue synthesis. At caloric intakes below the critical level the animal must resort to burning protein for purely energy purposes. As a consequence, the restriction of caloric intake below the critical point has the same net effect as a restriction of the protein intake.

Fat and protein synthesis in the depleted animal are partly independent. At low caloric intake levels both fat and protein storage are proportional to the caloric intake. At caloric intake levels above the minimum necessary for maximum protein fabrication, fat and protein metabolism are essentially independent (Fig. 4). A partial independence of the fat and protein metabolism has been observed in human beings.[10] It was found that obese individuals on protein intakes of 1 to 1.5 Gm. per kilogram per day and on caloric intakes 30 per cent less than estimated basal needs lost weight but remained in nitrogen equilibrium. Nonobese individuals on such a regime not only lost weight, but also had negative nitrogen balances. Thus it appears that the normal organism requires a certain quantity of fatty tissue in addition to nonfat tissue; but excessive quan-

tities of fat are not only unnecessary but also probably undesirable and can be dispensed with without sacrificing more needed tissues. Some fat gain is necessary in the rehabilitation of depleted individuals for the purpose of building up energy reserves. Excessive fat gain is probably to be avoided since it appears to do little but place an extra physical strain upon the organism and in addition is economically wasteful. Therefore, *adequate* but not excessive caloric intake is the aim in convalescent and rehabilitation feeding.

The statement has been made that nitrogen *balance* can be maintained on a low caloric intake for short periods of time by giving relatively large amounts of protein.[3] Our findings agree with this. Thus at a low caloric intake (560 Cal.

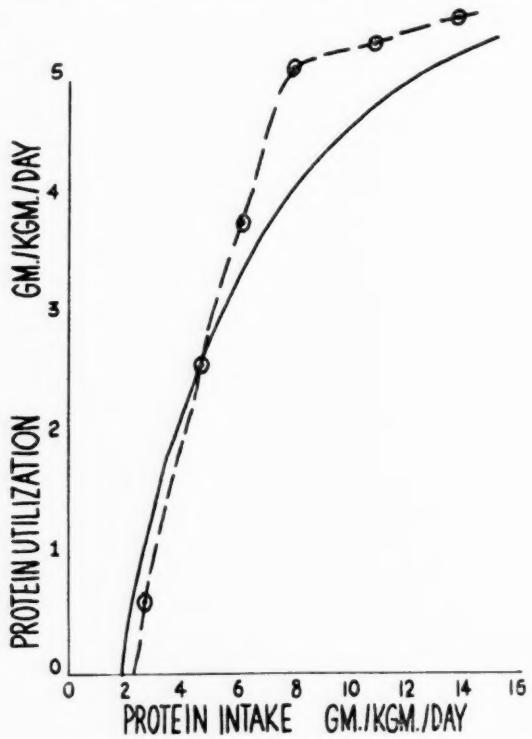

Fig. 6.—The rate of protein utilization for tissue synthesis at different levels of protein intake. —, Adult depleted rats; _ _, growing rats, after Barnes and coworkers.[13]

per square meter per day) and a protein intake of 9 Gm. per kilogram per day very low degrees of nitrogen utilization were found, similar to those obtained by Elman and associates[9] in dogs. But it is obvious from our observations that this is a highly inefficient procedure in terms of nitrogen utilization. Moreover, in a debilitated patient the desired result is not the achievement of mere nitrogen equilibrium, but rather the high degrees of nitrogen retention which go with the fabrication of significant quantities of new tissue.

Long ago it was found that the rate of gain of body weight in growing animals increases as the protein intake is increased.[11] Forbes and co-workers[12] investigated this problem further with careful studies of nitrogen and energy

metabolism. Unfortunately their lowest protein intake level was 7 Gm. per kilogram per day so that they have no data on the important lower levels of intake. More recently this problem has been reinvestigated by Barnes and colleagues.[13] These investigators fed protein at various intake levels to growing rats, keeping the energy intake approximately constant. They did nitrogen determinations on the carcasses. Their data, recalculated and plotted in Fig. 6 along with the observations from Experiment 2 described here, are in excellent agreement with ours derived from the adult protein-depleted rat.

This agreement becomes all the more significant when one considers the fact that the animals used in the two experiments differed both in age and size, one group being protein-depleted. A fundamental relationship appears to exist between the intake level of protein and the rate of protein utilization for the fabrication of tissue in the growing animal and in the adult protein-depleted animal undergoing repletion. Putting all of the foregoing facts together, we infer that the fabrication of a kilogram of new tissue in a growing animal and the reconstruction of a kilogram of tissue in an animal which has lost tissue due to protein deficiency demand similar quantities of structural material and similar constructing energies.

This does not imply that the quantitative requirements and the behavior of tissue synthesis in relation to protein intake are the same under all conditions, because certain things may modify the rate of utilization of protein. Thus as an animal approaches its growth maximum, the rate of utilization of protein for synthesis of new tissue declines. In the face of injuries such as burns, fractures, or infections there also is a marked decrease in the rate of tissue synthesis.[14] But, other things being equal, the rate of utilization of a high quality protein for synthesis of new tissue increases with increasing levels of protein intake providing the caloric intake is adequate to supply both the basal energy needs of the organism and the energy needed for synthesis including that stored in new tissue.

The question arises as to how far these data derived from the rat are applicable to human beings. In a later paper evidence will be presented demonstrating the fundamental similarity of the protein fabricating mechanisms in these two groups of mammals.

SUMMARY AND CONCLUSIONS

Experiments are described which were designed to elucidate the interrelationships between protein intake and energy intake as they affect the utilization of protein for tissue synthesis in standard protein-depleted rats.

The following conclusions are drawn:

Restriction of the caloric intake below a certain critical level restricts the utilization of ingested protein for the fabrication of tissue. Furthermore, an increase in caloric intake above this critical level does not augment the rate of utilization of a given quantity of protein above the maximum attainable with the particular protein fed under the particular circumstances of feeding (that is, level of protein intake, needs of animal, and so on). This critical level ap-

pears to be approximately 1,240 Cal. per square meter per day, and constitutes the energy necessary to cover the needs for maintenance, storage, tissue synthesis, and waste.

Increasing the caloric intake above the critical level needed for maximal rates of protein synthesis results in increased rates of body weight gain due largely to deposition of fat.

With an adequate caloric intake the rate of utilization of protein is a function of the level of intake and, in general, utilization rises with increasing intake.

The fabrication of a kilogram of tissue in a growing rat and the reconstruction of a kilogram of tissue in the adult protein-depleted rat demand the same quantities of structural material and similar constructing energies.

REFERENCES

1. Wissler, R. W., Woolridge, R. L., Steffee, C. H., and Cannon, P. R.: The Relationship of the Protein Reserves to Antibody Production, J. Immunol. **52**: 267, 1946.
2. Benditt, E. P., Straube, R. L., and Humphreys, E. M.: The Determination of Total Circulating Serum Proteins and Erythrocytes in Normal and Protein-Depleted Rats, Proc. Soc. Exper. Biol. & Med. **62**: 189, 1946.
3. Addis, T., Poo, L. J., Lew, W. W., and Yuen, D. W.: Gravimetric Methods for the Determination of Total Body Protein and Organ Protein, J. Biol. Chem. **113**: 497, 1936.
4. Lee, M. O.: Determination of the Surface Area of the White Rat With Its Application to the Expression of Metabolic Results, Am. J. Physiol. **89**: 24, 1929.
5. Osborne, T. B., Mendel, L. B., and Ferry, E. L.: A Method of Expressing Numerically the Growth-Promoting Values of Proteins, J. Biol. Chem. **87**: 223, 1919.
6. White House Conference on Child health and Protection: Growth and Development of the Child, Part III, Section I, p. 68, Century Co., Washington, 1932.
7. Altshuler, S. S., Hensel, H. M., Hecht, P., and Pursley, R.: Maintenance of Nitrogen Equilibrium by Intravenous Administration of Amino Acids, Arch. Int. Med. **70**: 749, 1942.
8. Bosshardt, D. K., Paul, W., O'Doherty, K., and Barnes, R. H.: The Influence of Caloric Intake on Growth Utilization of Dietary Protein, J. Nutrition **32**: 641, 1946.
9. Elman, R., Davey, H. W., and Kiyasu, R.: Nitrogen Balance on a Restricted Caloric Intake, J. Lab. & Clin. Med. **30**: 273, 1945.
10. Keeton, R. W., and Dickson, D.: Excretion of Nitrogen by Obese Patients on Diets Low in Calories, Containing Varying Amounts of Protein, Arch. Int. Med. **51**: 890, 1933.
11. Osborne, T. B., and Mendel, L. B.: The Comparative Nutritive Value of Certain Proteins in Growth and the Problem of the Protein Minimum, J. Biol. Chem. **20**: 351, 1915.
12. Forbes, E. B., Swift, R. W., Black, A., and Kahlenberg, O. J.: The Utilization of Energy Producing Nutriment as Affected by Individual Nutrient Deficiences. III. The Effects of the Plane of Protein Intake, J. Nutrition **10**: 461, 1935.
13. Barnes, R. H., Bates, M. J., and Maack, J. E.: The Growth and Maintenance Utilization of Dietary Protein, J. Nutrition **32**: 535, 1946.
14. Cuthbertson, D. P.: Protein Metabolism, Brit. M. Bull., **2**: 207, 1944.

THE DYNAMICS OF PROTEIN METABOLISM

II. The Relationship Between the Level of Protein Intake and the Rate of Protein Utilization by Protein-Depleted Men and Rats

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INTRODUCTION

TODAY, perhaps as never before, there is urgent need of more knowledge concerning basic principles of nutrition. Speedy rehabilitation of underfed persons with only limited quantities of available food demands the most judicious use of the food supply. To accomplish this we must know what part the major components of the diet, principally protein and energy, play in the fabrication of body tissue.

In a previous paper[1] we examined the effects of varying caloric and protein intakes upon the utilization of dietary protein for tissue synthesis by protein-depleted and growing rats. It was demonstrated that on an otherwise adequate diet a certain minimum level of caloric intake at a given protein intake level was needed to insure maximum tissue synthesis. Above this minimum level, increases in the caloric intake did not further augment protein utilization. Furthermore, with an adequate caloric intake the rate of protein utilization increased with increasing protein intake. These relationships were found to be similar in the growing rat and in the adult depleted rat during repletion. Such observations led us to examine data from the literature on human beings and to compare them with our data on rats. After doing so, it was found necessary to extend the rat experiments to include protein intake ranges commonly used and feasible in human feeding.

The following evidence demonstrates that animal experiments can be used as qualitative and quantitative guides in elucidating the problems of human nutrition providing the appropriate common denominators are employed in making the extrapolations from one species to another. Furthermore, with the combined data available on men and animals it is possible to attack intelligently the problem of constructing more efficient diets for starved and convalescing human beings.

METHODS AND MATERIALS

Observations on human beings providing simultaneous data on body weight, height, caloric intake, protein intake, protein source, nitrogen retention, and the degree of weight loss were sought from the literature. Although most such studies deal with diseased indi-

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viduals, one study was found[2] which dealt with the rehabilitation of otherwise healthy individuals suffering from severe inanition. Many otherwise good data had to be discarded because one or more important measurements were absent, particularly those of body weight and height. Moreover, many reports had very brief and incomplete accounts of diet composition.

Before we could compare the observations on rats with those on men it was necessary to have some common units of measurement. Obviously one could not compare directly the daily nitrogen excretion for a man of the order of 10 Gm. with that of a rat of the order of 0.1 gram. Expressed in absolute terms, each of these values has significance only in relation to the particular animal from which it was obtained. Units which compare quantities on the per animal basis are biologic units in a sense, but are not broad units which admit comparisons between animals of such widely different size as a rat and a man, nor even between large and small animals of the same species.

Assuming that the basic mechanisms of protein synthesis are the same for all of the mammalian species, upon what rational basis can we compare them? It has been demonstrated that energy consumption is approximately proportional to the surface area for a wide variety of mammals.[3] Basal nitrogen excretion in several mammalian species including man has been demonstrated to be proportional to the basal energy requirement.[4] Therefore it appears reasonable to compare the maintenance nitrogen requirements of different animals on the basis of surface area. Such a procedure is not necessarily valid when we are dealing with the construction of new tissue or the reconstruction of depleted tissue. What is the reasonable basis of comparison for feeding in this situation? The ingestion of protein under the conditions of synthesis and storage of new protoplasm falls in a class with many other chemical reactions. The chemist has long expressed additions to synthetic systems in terms of grams, moles, or equivalents of chemical substances per unit of fluid volume or mass. Viewing a kilogram of active protoplasm as such a chemical system, it appears rational to designate additions of protein offered to the system as so many grams per kilogram per day and likewise to compute protein retained and incorporated into the system in similar terms.

Energy intake is another measurement which must be reduced to common terms for comparative purposes. There is an old precedent for this in that basal metabolism has been expressed for many years in terms of calories per square meter of body surface per unit of time. We will not now discuss the significance of this relationship (see Brody[3]), but we have assumed its validity and computed the caloric intakes as calories per square meter of body surface per day (Cal. per square meter per day). For computation of the surface area of rats, Lee's formula[5] was used, and for the human being, the tables of Dubois.[6]

In the experimental observations, adult male albino rats (Sprague-Dawley) weighing initially 288 to 350 grams were depleted of 15 to 25 per cent of their body weight in a period of five weeks on an essentially protein-free diet.[7] At the end of the depletion period they were placed in individual metabolism cages. Five diets were constructed offering approximately 0, 1, 2, 3, and 4 Gm. of protein per kilogram of body weight per day. The basic composition of the diets was the same as that of the standard repletion diet used in this laboratory.[7] Isodynamic replacement of the carbohydrate by calculated quantities of protein furnished the required protein levels. The protein was a half-and-half mixture lactalbumin* and casein.† Fifteen grams of each ration were offered daily to each animal supplying it with 48 calories or between 1,200 and 1,400 Cal. per square meter per day. Five animals were used and the diets were rotated from day to day (Table II). The order of rotation for each animal was different. Daily collections of urine, feces, and waste food were made and the rats were weighed. The waste food was weighed

*Borden's No. 1542.
†SMACO, vitamin test, General Biochemicals Inc.

and pooled with the urine and feces for Kjeldahl nitrogen analyses. From these determinations, the diet analyses, and the food intake data the nitrogen balances were computed. Caloric intake was computed from the food intake using the conventional caloric equivalents.

A second experiment was performed using six animals and the 0, 1, and 4 Gm. per kilogram per day levels of protein intake along with three other diets of varying caloric value and bulk. The procedure used was similar to that described except that the feces were analyzed separately from waste food and urine. Throughout these experiments food waste was minimal, averaging 3 per cent and never over 7 per cent of the total offered.

OBSERVATIONS

A series of observations on the effects of various diets in the repletion of a group of severely starved but otherwise healthy men was published by von Hoesslin in 1919.[2] By comparison with normal height and weight tables[8] we estimated that the subjects had lost between 21 and 29 per cent of their body weight prior to the start of the tests. The repletion diets contained protein from both animal and vegetable sources of which 23 to 58 per cent was animal protein.

TABLE I. THE INFLUENCE OF VARYING CALORIC AND PROTEIN INTAKE UPON THE UTILIZATION OF PROTEIN BY MEN SUFFERING FROM SEVERE INANITION; DATA REPRESENT AVERAGES OF FOUR-, FIVE-, OR SEVEN-DAY PERIODS ON GROUPS OF FIVE MEN
(Recalculated from von Hoesslin: Arch. Hyg. 88: 147, 1919.)

PERIOD	BODY WEIGHT AT START OF PERIOD (KG.)	CALORIC INTAKE (CAL./M.²/DAY)	PROTEIN INTAKE (GM./KG./DAY)	PROTEIN UTILIZED (GM./KG./DAY)
6	50.0	1400	1.3	0.46
7	49.9	1500	0.6	−0.02
8	49.3	1700	0.5	0.02
1	45.0	1160	1.4	0.32
2	45.1	1700	1.3	0.36
3	44.8	1300	2.0	0.88
4	46.5	1440	2.1	0.86
1	48.2	1490	2.0	0.99
2	48.8	1650	2.6	1.34
3	49.0	2330	2.5	1.10
4	52.1	2580	3.5	2.04
5	51.5	1350	1.7	0.61
6	51.5	2650	1.7	0.60

Both caloric and protein intakes were varied and the nitrogen retention under the different conditions was observed. Groups of five men were fed each diet and the period used to test a single diet varied from four to seven days. A summary of the recalculated data is presented in Table I, and in Fig. 1 protein utilization is plotted against protein intake. The data in the table demonstrate that at a constant protein intake (1.7 Gm. per kilogram per day), increasing the caloric intake from 1,350 to 2,650 Cal. per square meter per day had no influence upon protein utilization. On the other hand, Fig. 1 demonstrates that protein utilization is proportional to protein intake over the range of protein intake studied.

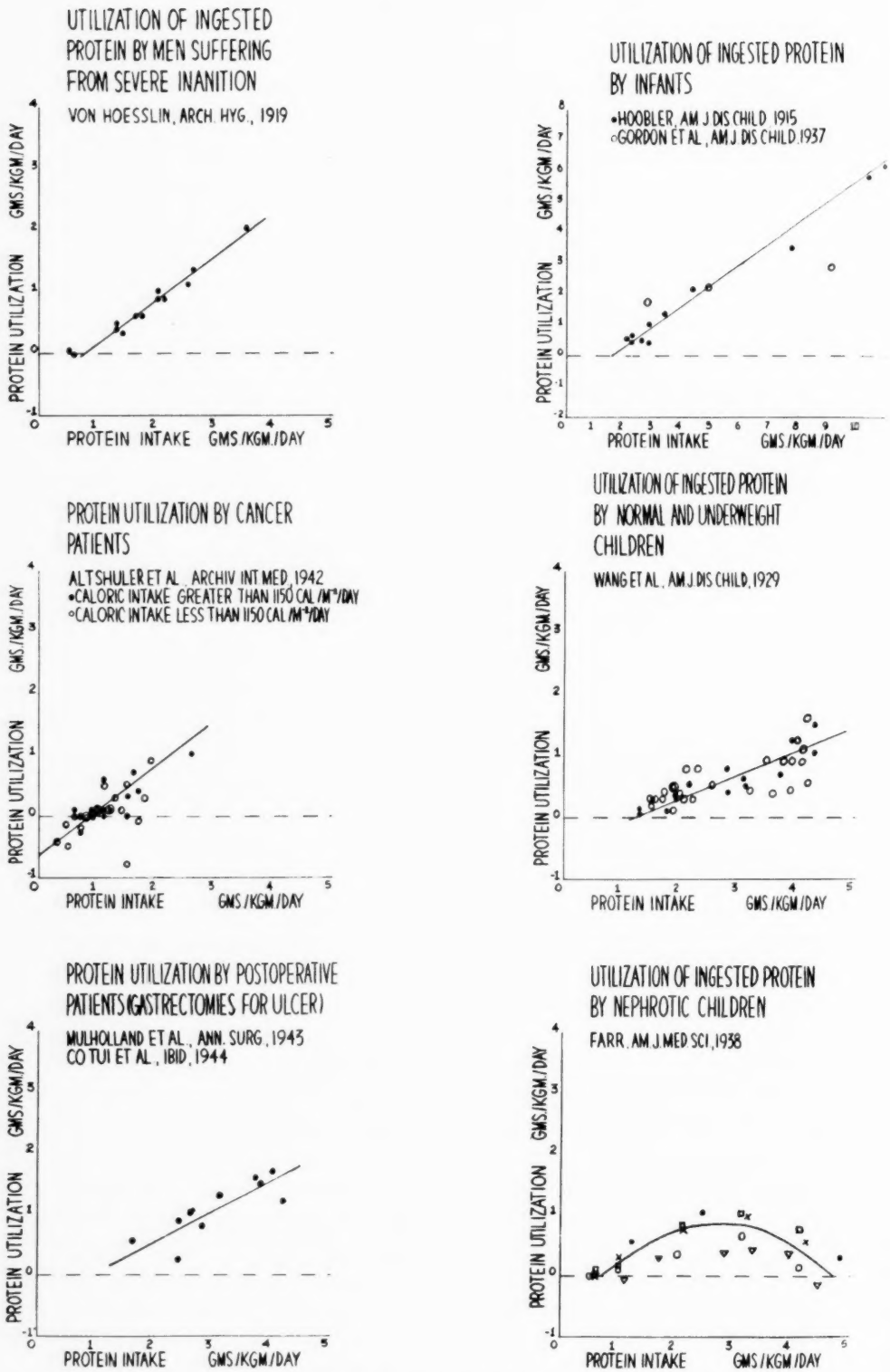
UTILIZATION OF INGESTED PROTEIN BY MEN SUFFERING FROM SEVERE INANITION
VON HOESSLIN, ARCH. HYG., 1919
PROTEIN UTILIZATION GMS./KGM./DAY
PROTEIN INTAKE GMS./KGM./DAY
UTILIZATION OF INGESTED PROTEIN BY INFANTS
•HOOBLER, AM. J. DIS. CHILD. 1915
○GORDON ET AL., AM. J. DIS. CHILD. 1937
PROTEIN UTILIZATION GMS./KGM./DAY
PROTEIN INTAKE GMS./KGM./DAY
PROTEIN UTILIZATION BY CANCER PATIENTS
ALTSHULER ET AL., ARCHIV INT. MED., 1942
•CALORIC INTAKE GREATER THAN 1150 CAL./M²/DAY
○CALORIC INTAKE LESS THAN 1150 CAL./M²/DAY
PROTEIN UTILIZATION GMS./KGM./DAY
PROTEIN INTAKE GMS./KGM./DAY
UTILIZATION OF INGESTED PROTEIN BY NORMAL AND UNDERWEIGHT CHILDREN
WANG ET AL., AM. J. DIS. CHILD., 1929
PROTEIN UTILIZATION GMS./KGM./DAY
PROTEIN INTAKE GMS./KGM./DAY
PROTEIN UTILIZATION BY POSTOPERATIVE PATIENTS (GASTRECTOMIES FOR ULCER)
MULHOLLAND ET AL., ANN. SURG., 1943
COTUI ET AL., IBID., 1944
PROTEIN UTILIZATION GMS./KGM./DAY
PROTEIN INTAKE GMS./KGM./DAY
UTILIZATION OF INGESTED PROTEIN BY NEPHROTIC CHILDREN
FARR, AM. J. MED. SCI., 1938
PROTEIN UTILIZATION GMS./KGM./DAY
PROTEIN INTAKE GMS./KGM./DAY

Fig. 1.—The influence of the level of protein intake on the rate of protein utilization by convalescent adult and growing human beings.

Observations were collected on a group of patients suffering from wasting diseases, principally cancer. Their usual hospital diet was supplemented with a protein hydrolysate (casein) administered intravenously, and nitrogen retention was studied. We have recalculated these data of Altshuler and co-workers[9] for nine cancer patients and plotted them in Fig. 1. The chart demonstrates that protein utilization increases proportionally, on the average, to the protein intake. Examination of the whole of the observations on the cancer patients by the method of partial correlations brings out the fact that over the entire range of caloric intake used, from 570 to 1,700 Cal. per square meter per day, caloric intake had no significant influence upon protein utilization; whereas protein utilization had a highly significant correlation with protein intake.

Patients convalescing from gastrectomies for peptic ulcer were fed through orojejunal tubes by Mulholland and Co Tui and colleagues.[10, 11] These patients received protein ranging from 2.4 to 4.2 Gm. per kilogram per day and caloric intakes between 1,100 and 2,700 Cal. per square meter per day. The protein source was an enzymatic hydrolysate of casein. In Fig. 1 the recalculated data on these patients have been plotted and demonstrate that protein utilization was proportional to the protein intake.

The protein metabolism of a healthy 2-month-old infant was studied by Hoobler.[12] The intake of cows' milk protein was varied from 2.2 to 10.9 Gm. per kilogram per day and the caloric intake was maintained between 1,040 and 1,240 Cal. per square meter per day with the exception of one period when it rose to 1,570 Cal. per square meter per day. The recalculated observations (Fig. 1) demonstrate that this young infant utilized ingested protein at a rate proportional to the protein intake. Gordon and associates[13] studied the protein metabolism of premature infants. Their data (Fig. 1) also demonstrate that the protein was utilized at a rate proportional to the level of protein intake and a little less efficiently at the higher levels of intake than it was utilized by the full term infant.

Wang and co-workers[14, 15] made extensive studies of the metabolism of normal and undernourished children. Their data show (Fig. 1) that the utilization of ingested protein nitrogen by both groups of children was proportional to the protein intake. The protein was mixed, but largely of animal origin. They found that children who were 20 per cent or more below the normal weight standards utilized the protein a little more efficiently than less underweight and normal children.[15] The absolute proportion of ingested protein retained by Wang's subjects averaged substantially less than that retained by the normal infant or the starved adults. Metabolism of nephrotic children at protein intake levels varying between 0.6 and 4.8 Gm. per kilogram per day was studied by Farr.[16] His data (Fig. 1) indicate that even these children utilized increasing quantities of protein as the protein intake was increased up to about 3 Gm. per kilogram per day, but beyond this as the intake was further increased the utilization fell sharply.

In Table II are the data derived from the experiments with rats, and in Fig. 2 the rate of protein utilization is plotted against the rate of protein intake. It is obvious that over the range of intake studied, the rate of utilization of a

TABLE II. PROTEIN UTILIZATION BY PROTEIN-DEPLETED RATS ON ADEQUATE CALORIC INTAKES

ANIMAL NUMBER	PERIOD NUMBER	BODY WEIGHT (GM.)	CALORIC INTAKE (CAL./M.²/DAY)	PROTEIN INTAKE (GM./KG./DAY)	PROTEIN UTILIZED (GM./KG./DAY)
		Experiment 1			
	1	285	1260	0.2	−0.8
	2	284	1240	1.1	−0.1
1	3	288	1190	2.1	0.9
	4	288	1230	3.7	2.5
	5	290	1210	4.5	3.2
	1	290	1230	3.9	2.0
	2	286	1240	0.2	−1.1
2	3	292	1230	1.2	−0.2
	4	290	1250	2.6	0.6
	5	296	1220	3.6	1.1
	1	282	1270	2.9	1.1
	2	280	1260	3.9	2.6
3	3	282	1250	0.2	−1.0
	4	285	1260	1.4	0.0
	5	288	1250	2.5	1.1
	1	236	1400	2.0	0.8
	2	234	1410	3.0	1.8
4	3	238	1410	4.1	2.9
	4	238	1380	0.3	−0.8
	5	240	1370	1.4	−0.2
	1	251	1380	1.1	−0.4
	2	248	1380	2.0	0.8
5	3	252	1370	3.0	1.6
	4	251	1370	4.7	2.8
	5	256	1350	0.3	−0.6
		Experiment 2			
	2	254	1260	4.7	3.0
6	5	276	1200	1.7	0.5
	6	253	1350	0.2	−0.9
	1	258	1360	0.2	−1.3
7	3	251	1270	4.2	2.7
	6	252	1270	1.9	0.5
	1	259	1250	1.9	0.4
8	2	259	1330	0.2	−1.5
	4	252	1270	4.8	2.8
	2	256	1260	1.9	0.7
9	3	256	1370	0.2	−0.9
	5	284	1180	4.8	2.7
	3	258	1270	1.9	0.2
10	4	255	1340	0.2	−1.4
	6	264	1260	4.6	2.7
	1	262	1260	4.6	2.7
11	4	251	1300	1.9	0.2
	5	286	1260	0.2	−1.0

high quality protein is proportional to the level of protein intake, providing the caloric intake is adequate. Comparison of these data with those of Experiment 2 in the preceding publication[1] shows that the two follow the same general pattern. Utilization of protein in the present experiments appears to be at a somewhat higher level than in the preceding one. This may be due in part to the fact that the periods of observation here were shorter, and that therefore the avidity of the tissues for protein remained closer to maximal; whereas with the fourteen-day experiments the growth potential was already significantly decreased toward the end of the period of observation.

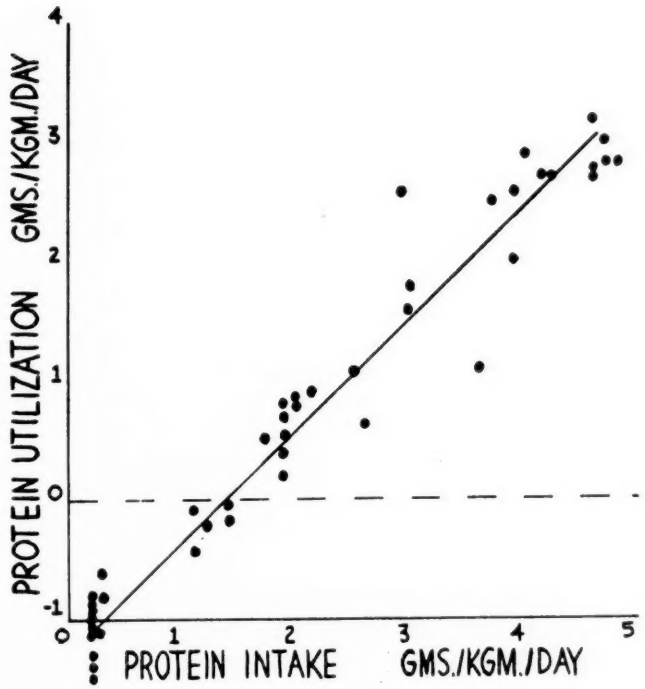

Fig. 2.—Utilization of ingested protein by protein-depleted rats on an adequate caloric intake at varying levels of protein intake.

DISCUSSION

The foregoing observations demonstrate that the protein-depleted and the growing animal, man or rat, given an adequate caloric intake utilize protein at a rate which is proportional to the rate of protein intake. For man, a similar pattern of reaction is apparent not only in convalescence from starvation alone, but also in convalescence from certain debilitating diseases. The similarity in the performance of the depleted rats and the starved men is strikingly evident when one compares Fig. 2 with Fig. 1.

At first glance the similarity in response to protein ingestion of these two species of mammals of such different size and external characteristics seems remarkable. These facts when compared with some of the many other observations on mammals appear less startling. They become not unique, but part of a great body of evidence accumulated over many years which points to the conclusion that the basic mechanisms underlying the organization and functioning of the tissues of the different species of animals are fundamentally similar. Let us examine a little of the evidence from the biochemical standpoint, having in mind, principally, a comparison of the rat and the human being. Body temperatures of rats and men are almost identical and their basal metabolic rates do not differ greatly.[4] In broader terms, the basal energy requirements of animals varying in size from the mouse to the elephant have been found to be proportional to a function of the body mass which approximates mathematically

the surface area.[3] The basal nitrogen excretion is related to the basal metabolism; thus rat, rabbit, guinea pig, man, and swine have been shown to excrete in urine an almost identical quantity of nitrogen per basal calorie per day.[4] The same amino acids, with the possible exception of histidine,[17] appear to be indispensable for the growth and maintenance of the rat and of man.[18, 19] Moreover we have evidence which suggests that the essential amino acids are needed in similar relative proportions in the two species for maintenance and for growth.[18] Viewing the matter broadly, there is a general quantitative, as well as qualitative, pattern into which rats and men as well as other mammals fit.

Von Noorden acknowledged the broad problem of the relationship of the level of protein intake and the caloric intake to the rate of repletion of lost tissue.[20] He outlined the types of experiments needed to settle the question, but presented evidence from only a single type of experiment, namely, that in which approximately the same quantity of protein per kilogram of body weight was fed per day to patients convalescing from debilitating infections while the caloric intake was varied. He concluded from these observations that high caloric intakes were more favorable than high protein intakes in inducing more rapid rates of rehabilitation.

Recently Keys has emphasized the efficacy of high caloric intakes to further rehabilitation in starved men.[21] He states that, "In relief feeding, calories are of overwhelming importance. Within reasonable limits every increase in calories is associated with an increased rate of recovery." He states further that, "Extra vitamins and proteins had very little apparent effect on the rate or course of recovery." The evidence which we have presented is not in accord with these statements, and demonstrates that while caloric intake is a factor, it is not the only factor. Moreover, with an adequate caloric intake, the level of protein intake and the quality of the protein become the limiting factors.

The role of caloric intake in problems of nutrition has been vastly overdrawn, for as Cathcart[22] has stated, "We do not live on calories. . . Calorie value is simply a very convenient physical standard for the assessment of diets, but merely because such a standard has proved of great utilitarian value there is no real justification for placing this standard as the foundation stone of hypotheses framed to offer an explanation of cellular activity. Many writers are obsessed with the idea of the calorie, forgetting that the organism is certainly not a heat engine. It is perfectly true that calories are a measure of heat, but it must not be forgotten that we do not consume actual heat units but only potential heat-giving substances which can eventually be degraded to the form of heat and be measured as such. The thermal aspect of nutrition is unduly stressed, for, while heat may be a necessary product of tissue activity, it is, after all, a by-product."

The aim when feeding sick, convalescent, starved, and growing individuals is not the mere maintenance of nitrogen equilibrium but the storage of significant quantities of protein as new tissue. The preceding observations demonstrate clearly that certain describable conditions must be satisfied to attain this goal. The conditions are as follows: (1) caloric intake must be adequate to cover needs for maintenance, physical exertion, tissue synthesis, storage, and

waste; (2) a protein of high biologic value must be fed; (3) the protein must be fed in excess of the quantity needed to maintain nitrogenous equilibrium, and in general the higher the level of protein intake the greater the rate of synthesis; (4) other dietary essentials (vitamins, minerals, and so on) must be adequate.

With these general propositions before us we are in a position to make more specific estimates of caloric and protein intakes for convalescence. But first we must clarify one further point. It has been customary to compute diets on the basis of total caloric content and protein content. The total caloric content has practically always included the caloric equivalent of the protein. In convalescence from starvation of any origin the maximal utilization of protein for tissue reconstruction is desired. Therefore it appears irrational to include protein in the computations both as a source of amino acids for tissue fabrication and as a source of fuel for energy-consuming reactions. The rational procedure is to feed protein as a source of building material and compute the energy value of the diet independent of its protein content on the basis of its carbohydrate and fat content.

An adequate caloric intake for a convalescent man can be estimated from considerations of the known basal requirements, requirements for physical exertion, protein synthesis, and waste. For an average sized man (surface area 1.7 square meters) afebrile and at bed rest the estimate is 2,600 calories per day or 1,500 Cal. per square meter per day.* Such an allowance represents not the absolute minimum, but is comparable to the standard ration used by us in many animal experiments which allows approximately 1,460 Cal. per square meter per day of nonprotein energy.

The animal data[1] indicate that a protein intake level of 10 Gm. per kilogram per day or more would provide better rates of protein synthesis than 2 or 4 Gm. per kilogram per day. Unfortunately it is not possible to feed adult human beings at this level. The reason for this becomes clear if one compares the per kilogram energy consumption of rats and men. The relatively large surface to mass ratio of the rat allows a 10 Gm. per kilogram per day protein intake to represent only a fraction of the total caloric intake (about one-eighth); whereas this level of protein intake would represent almost the entire daily caloric needs of a man. But the data indicate that a man can easily use up to 4 Gm. per kilogram per day. Protein intakes of the order of only 1 Gm. per kilogram per day will be ineffective in inducing significant degrees of protein storage. While this level of protein intake is the commonly accepted standard for *maintenance* of nitrogen equilibrium in a healthy individual, it is inadequate for the depleted

*The estimate was arrived at in the following manner. Basal requirements of a normal man are approximately 1,000 Cal. per square meter per day, and a starved man needs even less.[6] For the activity associated even with bed rest 30 per cent of the basal requirements were added. From thermodynamic considerations it has been shown[23] that the peptide bond contains a free energy equivalent of 7.5 calories per mole. Assuming only a 10 per cent efficiency in the biologic synthesis, it would take 83 calories to synthesize 100 Gm. of protein. To allow for the synthesis of 200 Gm. of protein with some excess, 200 calories were added for this factor. Exclusive of waste energy, the needs for the described situation are $1.7 \times 1,300 + 200 = 2,410$ calories. According to Rubner[24] about 8 per cent of the energy intake is lost in the feces; therefore 190 calories were added for this factor, totalling 2,600 calories.

individual in whom nitrogen must be stored. We cannot overemphasize the fact that there is a fundamental difference between the maintenance of the equilibrium state in the fully grown and healthy adult and the construction of new tissue in the depleted or growing individual. In order to obtain good rates of tissue synthesis under these latter conditions it is necessary to feed at protein intake levels of the order of 2 to 4 Gm. per kilogram per day. An examination of the data (Fig. 1) demonstrates that the higher protein intake levels have two advantages: first, a greater absolute rate of protein storage, and, second, a higher gross efficiency of protein utilization.

SUMMARY AND CONCLUSIONS

For the purpose of evaluating the respective roles of the level of caloric intake and the level of protein intake in the restoration of protein stores of depleted animals, we have presented further experiments on rats and a re-evaluation of observations from the literature on men. From these data the following conclusions are drawn: (1) In both groups of mammals with protein intakes ranging from 0.5 to 4 Gm. per kilogram of body weight per day the utilization of protein for tissue synthesis is proportional to the protein intake. (2) The rate of protein utilization is independent of the caloric intake at caloric intake levels above those which supply the needs for maintenance, synthesis, storage, and waste. The estimated nonprotein energy needs for an afebrile man at bed rest in the absence of other metabolic stimulants is approximately 1,500 calories per square meter of body surface per day. (3) Since the therapeutic goal is not the mere maintenance of nitrogen equilibrium but the fabrication of significant quantities of body protein, protein intake levels of 2 to 4 Gm. per kilogram per day are demanded for the rapid rehabilitation of a depleted person. At these levels there is both a higher gross efficiency of protein utilization and a greater absolute rate of protein gain.

REFERENCES

1. Benditt, E. P., Humphreys, E. M., Wissler, R. W., Steffee, C. H., Frazier, L. E., and Cannon, P. R.: The Dynamics of Protein Metabolism: I. The Interrelationship Between Protein and Caloric Intakes and Their Influence Upon the Utilization of Ingested Protein for Tissue Synthesis by the Adult Protein-Depleted Rat, J. Lab. & Clin. Med. **33**: 257, 1948.
2. Von Hoesslin, H.: Klinisches eigentumlichkeiten und Ernahrung bei schwerer inanition, Arch. Hyg. **88**: 147, 1919.
3. Brody, A. S.: Bioenergetics and Growth, New York, 1945, Rheinhold Publishing Corporation.
4. Lee, M. O.: Determination of the Surface Area of the White Rat With Its Application to the Expression of Metabolic Results, Am. J. Physiol. **89**: 24, 1929.
5. Smuts, D. B.: The Relationship Between the Basal Metabolism and the Endogenous Nitrogen Metabolism, With Particular Reference to the Estimation of the Maintenance Requirement of Protein, J. Nutrition **9**: 403, 1935.
6. Dubois, E. F.: Basal Metabolism in Health and Disease, Philadelphia, 1936, Lea and Febiger.
7. Frazier, L. E., Wissler, R. W., Steffee, C. H., Woolridge, R. L., and Cannon, P. R.: Studies in Amino Acid Utilization. I. The Dietary Utilization of Mixtures of Purified Amino Acids in Protein-Depleted Albino Rats, J. Nutrition **33**: 65, 1947.
8. McLester, J. S.: Nutrition and Diet in Health and Disease, Philadelphia, 1943, W. B. Saunders Company, p. 771.
9. Altshuler, S. S., Hensel, H. M., Hecht, P., and Pursley, R.: Maintenance of Nitrogen Equilibrium by Intravenous Administration of Amino Acids, Arch. Int. Med. **70**: 512, 1943.

10. Mulholland, J. H., Co Tui, Wright, A. M., and Vinci, V. J.: Nitrogen Metabolism, Caloric Intake and Weight Loss in Postoperative Patients, Ann. Surg. **117**: 512, 1943.
11. Co Tui, Wright, A. M., Mulholland, J. H., Caralba, V., Barelton, I., and Vinci, V. J.: Studies in Surgical Convalescence. I. Sources of Nitrogen Loss Following Gastrectomy and the Effect of High Amino Acid High Caloric Intakes on Convalescence, Ann. Surg. **120**: 99, 1944.
12. Hoobler, B. R.: The Protein Need of Infants, Being Metabolism Studies of a Two Months Old Infant Fed With Varying Proportions of Cows' Milk Protein, Am. J. Dis. Child. **10**: 153, 1915.
13. Gordon, H. H., Levine, S. Z., Wheatley, M. A., and Marples, E.: Respiratory Metabolism in Infancy and Childhood. XX. The Nitrogen Metabolism in Premature Infants, Am. J. Dis. Child. **4**: 1030, 1937.
14. Wang, C. C., Hawks, J. E., and Hays, B. R.: Metabolism of Undernourished Children. V. Protein Metabolism, Am. J. Dis. Child. **35**: 968, 1928.
15. Wang, C. C., and Kaucher, M.: Metabolism of Undernourished Children. VIII. The Effect of High and Low Protein Diets on the Excretion of Creatine, Creatinine and Ammonia, Am. J. Dis. Child. **38**: 468, 1929.
16. Farr, L. E.: The Assimilation of Protein by Young Children With the Nephrotic Syndrome, Am. J. M. Sc. **70**: 195, 1938.
17. Rose, W. C., Haines, W. J., Johnson, J. E., and Warner, D. R.: Further Experiments on the Role of Amino Acids in Human Nutrition, J. Biol. Chem. **148**: 457, 1943.
18. Rose, W. C.: The Nutritive Significance of the Amino Acids, Physiol. Rev. **18**: 109, 1938.
19. Cannon, P. R., and Others: Unpublished observations.
20. Von Noorden, C.: Metabolism, Physiology and Pathology. Vol. II. The Pathway of Metabolism, Chicago, 1907, W. T. Keener and Co., p. 70.
21. Keys, A.: Human Starvation and Its Consequences, J. Am. Dietet. A. **22**: 582, 1947.
22. Catheart, E. P.: The Influence of Fat and Carbohydrate on the Nitrogen Distribution in the Urine, Biochem. J. **16**: 747, 1922.
23. Schmidt, C. A.: The Chemistry of the Amino Acids and Proteins, Baltimore, 1938, Charles C Thomas, p. 865.
24. Rubner, M.: Quoted by Lusk, G.: The Science of Nutrition, Philadelphia, 1928, W. B. Saunders Company, p. 41.

THE DETERMINATION OF THE NITROGEN BALANCE INDEX OF A NEW LYOPHILIZED AMINO ACID PREPARATION IN PROTEIN-DEFICIENT PATIENTS

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PROTEIN products and protein hydrolysates intended for human nutrition have their principal usefulness in the repletion of protein-deficient patients. The criterion for clinical acceptability of such a product has been its ability to produce positive nitrogen balance in protein-deficient subjects. The quantity required for positive balance must be of the same order as that of the mixture of proteins in the common foods. If a much greater quantity is required it is recognized that the biologic value of the investigated product is relatively low, at least under the conditions of administration utilized.

Yet it has been increasingly clear from nitrogen balance experiments that human subjects with protein deficiency differ greatly from each other in the nitrogen intake of a particular protein required for nitrogen equilibrium, even when such subjects are chosen from an apparently homogeneous group. For example, in a nitrogen balance study of a casein hydrolysate in this laboratory[1] some subjects came into positive balance with a daily intake of 60 Gm. of amino acids, others with 90 Gm., and still others only after 120 grams. An occasional patient could not be brought into balance with as much as 135 Gm. of amino acids, equivalent to 16.9 Gm. of protein nitrogen. Comparable findings were obtained in the study of an oral lactalbumin derivative.[2] The calculation of the *average* requirement of a particular protein in experiments such as these is justifiable only if the number of experiments is large and if the deviations from the average are not too great. The utilization of such averages for comparison of one product with another is obviously fraught with danger.

Recent feeding experiments with protein-depleted dogs by Allison and co-workers[3, 4] have demonstrated that even laboratory animals under careful supervision show wide variations in protein requirements for nitrogen equilibrium. These investigators have been able to show that the nitrogen intake at equilibrium depended upon the nitrogen excretion on a protein-free diet, the latter excretion being a measure of the so-called endogenous protein metabolism. The lower the nitrogen excretion (NE_0) on the protein-free diet, the lower was the requirement for equilibrium. If the nitrogen balance (NB) obtained on successive nitrogen intakes, the latter being calculated as absorbed nitrogen (AN), was plotted against the absorbed nitrogen, a curve was obtained which in the region of negative and low positive nitrogen balance was a straight line.

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The equation for this relationship was $NB = K\ (AN) - NE_0$. The value K was the slope of the line and it was found to be a measure of the biologic value of the protein used in the study. It tended to be a constant for any given protein even when the nitrogen intake at equilibrium varied considerably. Thus the calculation of K appeared to be a much better characterization of the biologic value of the protein than the nitrogen requirement at nitrogen equilibrium. The values for K were usually less than 1; the nearer to 1 they were, the greater the fraction of absorbed nitrogen that was retained. Casein, for example, was found to have a K value of about 0.80. Allison has called K the nitrogen balance index.

It appeared to us, during the course of a study of the clinical acceptability of a new casein hydrolysate prepared for parenteral use, that the methods and equation of Allison might be applicable to nitrogen balance studies in human subjects. The present investigation was therefore undertaken to find out whether the nitrogen balance index of this amino acid preparation tended to be constant in the nitrogen balance experiments with twenty patients with chronic protein deficiency and whether such an index was a better criterion of biologic value than the nitrogen intake at nitrogen equilibrium.

METHODS

The study was carried out in a metabolic unit staffed twenty-four hours a day by nurses specifically chosen for this function. The urine specimens were collected for each twenty-four hour period in closed bottles containing toluene and were sent each day to the laboratory for analysis. The subjects were under constant supervision to avoid the loss of specimens. A further check was the determination of the daily creatinine excretion.

Since none of the twenty patients chosen had any difficulty taking oral alimentation, this avenue was utilized as a means of furnishing an adequate intake of calories from carbohydrate and fat. The oral dietary was designed to be virtually free of protein so that the only significant source of nitrogen would be the parenterally injected amino acids. However, the food orally ingested could not be made rigidly free of nitrogen, as might be possible with laboratory animals or volunteer human subjects. The dietary consisted of a carbohydrate drink,* tapioca and cornstarch puddings, orange juice, tomato juice, and a puréed leafy vegetable. Since the amount of nitrogen in the foods chosen was low (between 350 and 500 mg.) and since it was derived from proteins of poor absorbability and poor biologic value, the error involved in the inclusion of this nitrogen source was probably of no consequence. It was probably no higher than the nitrogen lost in perspiration, even though the studies were carried out in cool weather, and was therefore not included in the calculation. The oral carbohydrate and fat plus the intravenously injected dextrose furnished approximately 2,000 calories per day, but slight variation above or below this quantity could not be avoided.

The amino acid preparation under study was a casein acid hydrolysate fortified with tryptophane and prepared as a lyophilized solid in 850 c.c. flasks.† The nitrogen content and the percentage composition of essential amino acids in the preparation are shown in Table I. A substantial portion of the glutamic and aspartic acids present in the original hydrolysate had been removed by a special process. Each flask contained 60 Gm. of the lyophilized solid which could be reconstituted to a perfectly clear solution with water, physiologic saline or dextrose-saline solutions. The solution could be made up in 5,

*The carbohydrate used was Cartose, furnished by H. W. Kinney and Sons, Columbus, Ind.

†The product, called Amino Acids—I. C., Lyophilized, was furnished by The Biochemical Division, Interchemical Corporation, Union, N. J.

7.5, or 10 per cent concentrations of the amino acids. Most of the injections were made with a 5 per cent solution at a rate between 50 and 60 drops per minute in order to make sure that there would be no vomiting. But even when the 10 per cent solution was utilized in these and other experiments, if this rate was not exceeded nausea or vomiting seldom occurred. Repeated injection in the same vein tended to produce thrombophlebitis, as has been found with other protein hydrolysate preparations used. But this circumstance could be mitigated by frequent change of the veins utilized and by starting with the most peripheral veins and proceeding cephalad. The needles used were No. 19 gauge and were introduced with a minimum of trauma to the veins.

TABLE I. CHEMICAL ANALYSIS OF AMINO ACID PREPARATION USED IN THIS STUDY (AMINO ACIDS, I.C., LYOPHILIZED)

	(%)
Nitrogen	
Total	13.4
Alpha amino	10.0
% Alpha amino nitrogen of total nitrogen	75.0
Essential amino acids (by microbio-assay)	
Arginine	3.8
Histidine	2.7
Isoleucine	7.4
Leucine	10.6
Lysine	8.5
Methionine	3.0
Phenylalanine	5.5
Threonine	5.0
Tryptophane	0.5*
Valine	7.3
Moisture	1-2.0
Ash	0.9

*One per cent DL-tryptophane added.

All patients were first placed on the protein-free diet for a period of three days or more to establish the endogenous nitrogen excretion level. Then daily infusions of amino acids were given in addition to the oral alimentation in quantities known not to produce positive balance. After three or four days of this regimen, the quantity of amino acids was further increased and maintained at the new level for three or more days. With successive increases a nitrogen balance was achieved that was barely positive. Further increases were then made at a more rapid rate to determine, if possible, the maximal level of utilization.

Careful attention was paid to the vitamin requirements of the patients. Ascorbic acid was given in doses of 500 mg. per day; thiamin and other members of the vitamin B complex were given as Betalin Complex,* one c.c. of which provided 5 mg. thiamin chloride, 2 mg. riboflavin, 75 mg. nicotinamide, 2.5 mg. calcium pantothenate, and 5 mg. pyridoxine hydrochloride.

Quantitative analyses of the nitrogen content of blood, urine, feces, and foods as well as that of the hydrolysate were carried out by the photometric micro-Kjeldahl methods described by Hoffman and Osgood.[5] They were frequently checked by macro-Kjeldahl determinations involving distillation and titration. For stool analysis, the preparations were first homogenized in water by the addition of concentrated sulfuric acid, as suggested by Peters and Van Slyke.[6] Plasma volumes were estimated from a single ten-minute plasma sample after the injection of 10 mg. of Evans blue as described by Gregerson.[7] A calibration curve was made in each analysis with the control serum. To avoid variations in the fat content of the control and dye-containing samples, the tests were made at least twelve hours after the previous meal or infusion of amino acids. Hematocrit determinations were made on heparinized blood by the method of Wintrobe and Landsberg.[8] Urine and stool analyses were made daily; blood analyses including plasma volume estimations were made usually at weekly intervals, at which time the patient was weighed.

*Furnished by Eli Lilly & Company, Indianapolis, Ind.

TABLE II. NITROGEN BALANCE STUDIES IN TWENTY PROTEIN-DEFICIENT PATIENTS; DETERMINATION OF MINIMAL AMINO ACID INTAKE PRODUCING NITROGEN BALANCE

PATIENT	AGE (YR.)	DIAGNOSIS	WEIGHT (KG.)	AMINO ACID INTAKE (GM./DAY)	N INTAKE (GM./DAY)	N INTAKE (MG./KG./DAY)	N EXCRETION (GM./DAY)	N BALANCE (GM./DAY)	N BALANCE (MG./KG./DAY)	N REQUIREMENT FOR EQUILIBRIUM (INTERPOLATED) (MG./KG./DAY)	K (FROM FIG. 1)
J. V. 1	61	Cancer of esophagus	45.9	0	0	0	1.8	−1.8	−40	50	0.97
				26	3.4	73	2.0	+1.4	+30		
				52	6.7	146	2.2	+4.5	+98		
				82	10.8	271	8.0	+2.8	+61		
S. D. 2	62	Gastric ulcer	55.4	0	0	0	4.0	−4.0	−72	158	0.46
				29	3.7	67	5.7	−2.0	−36		
				58	7.4	134	8.1	−0.7	−13		
				87	11.1	201	10.0	+1.1	+20		
				116	14.8	268	14.2	+0.6	+11		
				180	22.7	411	18.7	+4.0	+72		
F. L. 3	48	Cancer of esophagus	62.0	0	0	0	5.4	−5.4	−87	116	0.74
				25	3.3	53	7.9	−4.6	−74		
				50	6.6	106	5.1	+1.5	+24		
				70	9.3	150	6.7	+2.6	+42		
				82.5	10.7	173	8.1	+2.6	+42		
				160.5	20.4	329	14.3	+6.1	+98		
J. Z. 4	65	Cancer of esophagus	41.6	0	0	0	7.8	−7.8	−188	340	0.54
				32.9	4.1	99	6.5	−2.4	−58		
				70.5	8.8	212	9.9	−1.1	−26		
				94.0	11.0	264	12.8	−1.8	−43		
				150.0	19.8	476	17.3	+2.5	+60		
				200.0	26.4	635	18.8	+7.6	+183		
H. A. 5	59	Malnutrition; ?aplastic anemia	47.8	0	0	0	5.3	−5.3	−111	185	0.61
				26.5	3.4	71	6.6	−3.2	−67		
				53.0	6.8	142	7.5	−0.7	−15		
				79.5	10.2	213	9.3	+0.9	+19		
				106.0	13.6	284	10.7	+2.9	+61		
R. S. 6	39	Malnutrition	52.7	0	0	0	4.3	−4.3	−82	150	0.49
				32.9	4.1	78	5.8	−1.7	−32		
				70.5	8.8	167	9.0	−0.2	−4		
				94.0	11.0	209	9.9	+1.1	+21		
				150.0	19.8	376	13.2	+6.6	+125		
				200	26.4	501	13.2	+13.2	+250		
B. O. 7	65	Cancer of esophagus	58.9	0	0	0	9.9	−9.9	−168	—	—
				50	6.6	112	12.4	−5.8	−98		
				25	3.3	56	7.6	−4.3	−73		
				50	6.6	112	8.9	−2.3	−39		
				70	9.4	152	16.2	−6.8	−115		
				82	10.7	182	13.2	−2.5	−42		
M. G. 8	75	Splenic anemia	43.5	0	0	0	6.8	−6.8	−156	155	1.02
				29.2	3.7	84	5.9	−2.1	−48		
				58.4	7.3	168	6.8	+0.5	+11		
				87.6	11.0	251	9.8	+1.2	+28		
				116.8	14.6	335	13.5	+1.1	+25		
				147.0	19.0	435	16.8	+2.2	+51		
L. H. 9	45	Cirrhosis with ascites	57.9	0	0	0	1.5	−1.5	−26	42	0.52
				26	3.3	57	3.1	+0.2	+3		
				52	6.7	115	3.2	+3.5	+60		
				104	13.4	231	6.6	+6.8	+117		
A. B. 10	56	Chronic ulcerative colitis; nontoxic goiter	39.6	0	0	0	3.5	−3.5	−88	197	0.44
				28.5	3.6	99	4.7	−1.1	−28		
				57.0	7.2	182	7.9	−0.7	−18		
				85.5	10.8	273	9.5	+1.3	+33		
				113.0	14.3	364	11.9	+2.4	+61		

(Continued on following page.)

TABLE II.—CONT'D

PATIENT	AGE (YR.)	DIAGNOSIS	WEIGHT (KG.)	AMINO ACID INTAKE (GM./DAY)	N INTAKE (GM./DAY)	N INTAKE (MG./KG./DAY)	N EXCRETION (GM./DAY)	N BALANCE (GM./DAY)	N BALANCE (MG./KG./DAY)	N REQUIREMENT FOR EQUILIBRIUM (INTERPOLATED) (MG./KG./DAY)	K (FROM FIG. 1)
M. M. 11	66	Leg ulcers	59.9	0	0	0	6.4	−6.4	−107	140	0.79
				30	3.8	64	7.0	−3.2	−53		
				44	5.5	92	6.6	−1.1	−18		
				60	7.7	128	9.7	−2.0	−33		
				70	8.8	148	8.9	−0.1	−2		
				90	11.5	192	8.9	+2.6	+43		
				174	22.1	369	17.8	+4.3	+72		
L. M. 12	60	Huge thigh ulcer	41.5	0	0	0	5.2	−5.2	−125	146	0.84
				27.5	3.5	84	5.6	−2.1	−51		
				44.0	5.5	133	4.9	+0.6	+18		
				55.0	7.1	171	6.3	+0.8	+19		
				71.5	9.0	217	9.0	0	0		
				95.0	12.1	292	11.2	+0.9	+22		
P. M. 13	61	Wound separation; bowel obstruction	53.2	0	0	0	5.4	−5.4	−101	140	0.73
				28	3.6	68	5.2	−1.6	−30		
				45	5.7	107	6.6	−0.9	−17		
				56	7.2	136	6.1	+1.1	+21		
				84	10.8	204	8.3	+2.5	+47		
				112	14.3	269	12.3	+2.1	+39		
A. T. 14	52	Infected mastectomy wound	39.8	0	0	0	3.0	−3.0	−75	125	0.58
				27.5	3.5	88	3.2	+0.3	+8		
				40.0	5.0	126	5.3	−0.3	−8		
				50.0	6.3	158	5.4	+0.9	+23		
				75.0	9.5	239	7.0	+2.5	+63		
				108.0	13.4	340	10.4	+3.0	+75		
J. W. 15	68	Cancer of rectum	43.2	0	0	0	2.4	−2.4	−56	115	0.49
				30	4.0	92	4.3	−0.3	−7		
				40	5.3	123	5.7	−0.4	−9		
				50	6.6	153	6.0	+0.6	+14		
				59	7.9	183	7.6	+0.3	+7		
				118	15.8	366	11.8	+4.0	+93		
J. T. 16	72	Leg ulcer	55.2	0	0	0	4.7	−4.7	−85	100	0.98
				30	4.0	73	4.4	−0.4	−7		
				47	6.5	117	5.5	+1.0	+18		
				59	8.1	146	5.6	+2.5	+45		
				118	16.2	292	11.9	+4.3	+78		
				180	23.9	433	13.8	+10.1	+183		
M. T. 17	65	Femoral hernia	50.0	0	0	0	1.5	−1.5	−30	48	0.63
				20	2.7	54	2.5	+0.2	+4		
				56	7.6	152	4.8	+2.8	+56		
				62	8.5	170	4.8	+3.7	+74		
				110	14.6	290	12.2	+2.4	+48		
V. E. 18	48	Essential hypertension	39.4	0	0	0	2.6	−2.6	−66	90	0.73
				30	4.0	102	3.4	+0.6	+15		
				43	5.9	150	4.2	+1.7	+43		
				62	8.5	216	6.1	+2.4	+61		
				110	14.6	371	12.1	+2.5	+63		
T. O. 19	47	Gastro-jejuno-colic fistula	47.3	0	0	0	2.9	−2.9	−61	98	0.62
				34	4.5	95	2.7	+1.8	+38		
				57	7.5	159	6.6	+0.9	+19		
				114	15.0	318	8.1	+6.9	+146		
				171	22.5	477	15.1	+7.4	+156		
E. R. 20	58	Bronchiectasis	54.5	0	0	0	2.4	−2.4	−44	62	0.70
				28	3.7	68	3.8	−0.1	−2		
				55	7.3	134	4.2	+3.1	+31		
Average and standard deviations										129 ± 66 (51%)	0.68 ± 16 (24%)

RESULTS

Minimal Requirements for Positive Nitrogen Balance.—Positive nitrogen balance was achieved in nineteen of the twenty subjects studied. The data are shown in Table II. Patient 7, who had a carcinoma of the esophagus, had not come into positive nitrogen balance on an intake of 182 mg. nitrogen per kilogram per day. This patient might possibly have achieved positive balance at a higher level of intake, but he could not be kept on the study any longer. The remaining nineteen patients showed a wide variation in the intake needed for positive balance, ranging from 57 to 476 mg. nitrogen per kilogram daily. But these values had little meaning since the levels of positive balance achieved with these quantities varied considerably. Much more significant were the nitrogen intakes required for each nitrogen equilibrium. These quantities could

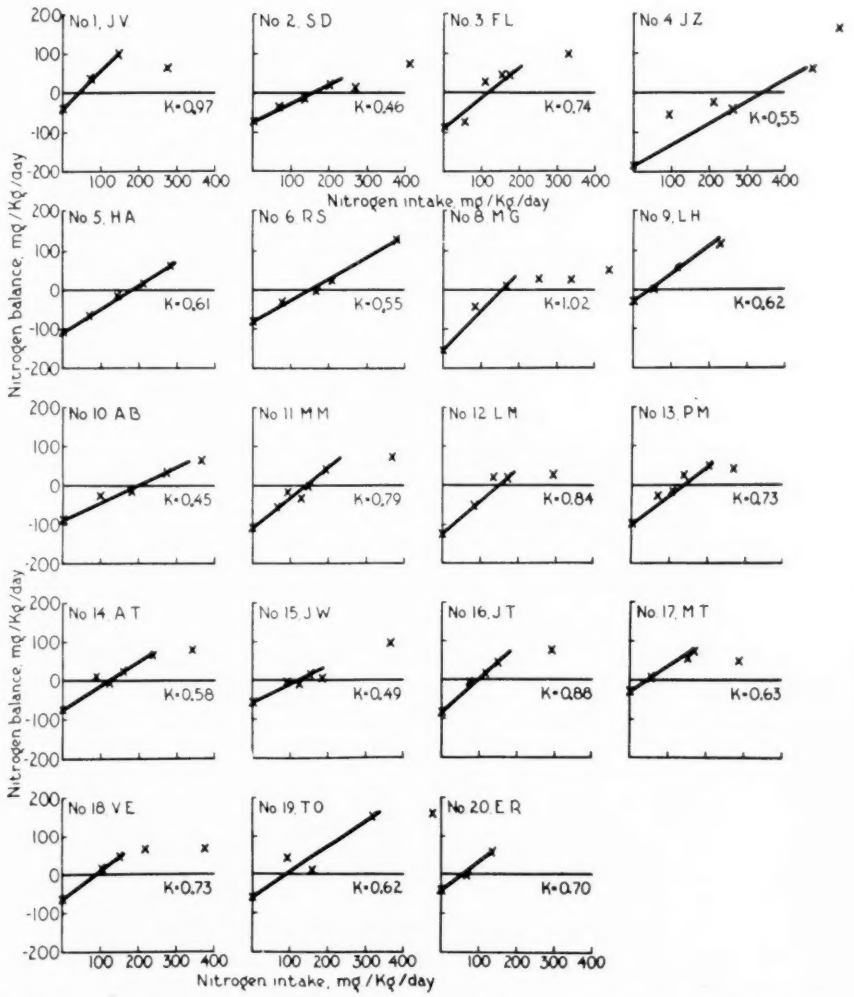

Fig. 1.—Nitrogen balance curves in nineteen subjects in whom positive nitrogen balance was achieved.

be estimated only by interpolation from the curves obtained by plotting nitrogen balance against nitrogen intake (Fig. 1). Of the nineteen curves, thirteen were reasonably good straight lines in the region of negative and low positive balance; the remaining six required some arbitrariness for the establishment of straight lines. The range of nitrogen intake levels required for nitrogen equilibrium in all nineteen cases was 42 to 340 mg. per kilogram per day. The arithmetic mean was 129 mg. and the standard deviation, 66 mg., which was 51 per cent of the mean. If only the thirteen good cases were chosen, the range was 42 to 197 mg. per kilogram per day, with a mean of 122 mg. and a standard deviation of 48 mg., or 39 per cent of the mean. Thus it was evident that though the range of nitrogen intakes required for nitrogen equilibrium was narrowed if the proper experiments were chosen, that range was still broad.

Nitrogen Balance Index.—As has been stated, thirteen of the nineteen nitrogen balance curves were straight lines in the region of negative and low positive nitrogen balance. Even the remaining six curves did not show great deviations from a straight line, but their exact slopes were difficult to determine.

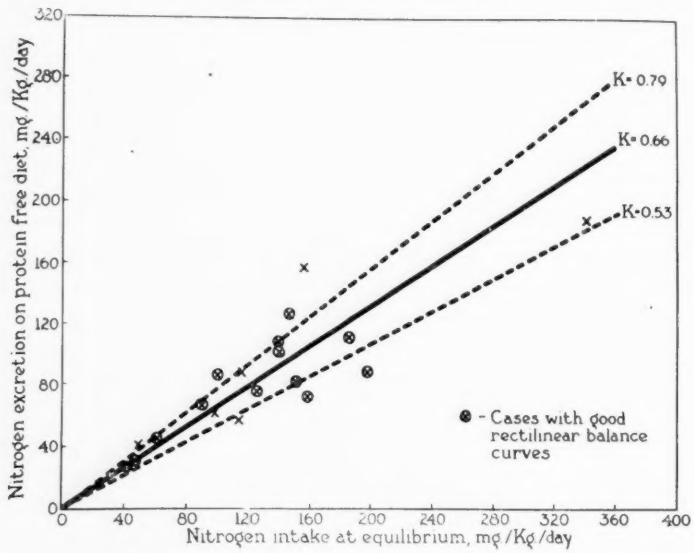

Fig. 2.—Relation of nitrogen intake at nitrogen equilibrium to nitrogen excretion on a protein-free diet. (K is the ratio of nitrogen excretion on a protein-free diet to the nitrogen intake at nitrogen equilibrium.)

These straight-line curves obeyed the equation shown earlier. (Nitrogen intake in these experiments was equal to absorbed nitrogen if the small amount of ingested nitrogen which was absorbed from the intestine was ignored.) Thus these experiments confirm in human subjects with protein deficiency produced by disease the findings of Allison and Anderson[3] for dogs with induced protein deficiency.

The K values for the equations of the curves shown in Fig. 1, which were the slopes of these curves and which were most easily calculated from the ratio of nitrogen excretion on a protein-free diet to the nitrogen intake at nitrogen

equilibrium, ranged from 0.45 to 1.02. The mean was 0.68 and the standard deviation was 0.16, or 24 per cent of the mean. In the thirteen cases with good straight-line curves, K ranged from 0.45 to 0.88 and the mean was 0.66, with a standard deviation of 0.13, or 20 per cent of the mean. In consideration of the difficulties of nitrogen balance studies in such patients as well as the many potential sources of error in the nitrogen analyses, the K values arrived at in these experiments showed remarkably small deviation from the average. These findings also offered corroboration of Allison's conclusion that the K values tended to be constant for any particular protein. This approximate constancy is graphically illustrated in Fig. 2 where nitrogen excretion on the protein-free diet has been plotted against the nitrogen intake at equilibrium. The scatter points are closely bunched along a straight line which has the slope of mean K value 0.66. The dotted lines demarcate the standard deviation of the K values. Both the group of thirteen good experiments and the six more doubtful ones fall equally well into the wedge within the boundaries of this standard deviation.

Fig. 2 also brings out the important corollary to Allison's equation, that there was no tendency to constancy of nitrogen intake at equilibrium but that that value depended upon the endogenous nitrogen metabolism as represented by the nitrogen excretion on a protein-free diet. The higher the latter, the higher was the nitrogen intake required to produce equilibrium. In this group of patients four showed remarkably low nitrogen excretions in the control protein-free periods (Patients 1, 9, 17, and 20). At first these low excretions were blamed on losses of specimens, but careful examination failed to reveal any such technical carelessness. Besides, the urine volumes in these cases were large, the specific gravities low, and the creatinine excretions generally constant. Furthermore, these four subjects showed K values very close to the average and the nitrogen balance curves were good approximations of straight lines. The low excretions were therefore probably genuine. The nitrogen intakes at equilibrium for these four patients were at unusually low levels of 42 to 62 mg. per kilogram per day. On the other hand, those patients with higher endogenous nitrogen excretions all showed higher intakes at equilibrium. None of this group had the unusually high excretions found by Co Tui and co-workers[9] in patients with burns or other conditions producing the so-called catabolic assault. If there were such patients, they likely would have shown much higher nitrogen intakes at equilibrium than seen in Fig. 1, if the K values were of the same order as the values found here.

The unusually high K value of 1.02 might represent a technical error, especially in the determination of the endogenous excretion. An occasional subject when placed on the protein-free, high-carbohydrate, high-fluid diet excreted relatively large quantities of nitrogen for several days before reaching a stable excretion. Thus with only a three- or four-day control period the patient might still not show the true endogenous excretion. If such a point was used as the pivotal point for drawing the straight line curve, the slope would tend to be too steep, making K too high. If, however, this value was not due to error, it

meant that in that particular patient the addition of protein to the diet spared endogenous metabolism. This phenomenon has been suggested by Allison in some of his balance studies with dogs.

Maximal Tolerance of the Hydrolysate.—Of the nineteen subjects carried to the point of good positive balance, all but two finally received more than 100 Gm. of the protein hydrolysate daily for at least three days. As much as 147 Gm. were given daily to eight patients and 200 Gm. to two patients for three days. No effort was made in these experiments to extend the injections beyond this period, and it is likely that some extension could have been made before the development of findings of intolerance such as nausea, vomiting, anorexia, or progressive thrombophlebitis. It may be significant that the greatest weight gains occurred in the patients who received as high as 200 Gm. of amino acids daily. When the 10 per cent solution was used, the injection of 60 Gm. usually required two to three hours for administration. Thus 180 Gm. could be injected, if need be, in six to nine hours and there would still be time for additional saline or glucose injection without infringement on the period of sleep.

The nitrogen balance curves in the region of high intake were not so steep as those in the region of negative or low positive balance. In fact, in several cases the curves became horizontal, indicating a ceiling of nitrogen retention. In one subject, however, a positive nitrogen balance of 13 Gm. was achieved and in another, 10 grams. In the remaining patients the positive nitrogen balance increased only slowly with higher intakes, which posed the problem of whether the increased quantities injected produced enough additional retention of nitrogen to warrant the effort.

It has been maintained[19] that parenterally injected amino acids so reduce the appetite as to cause a diminution of the oral intake of food. This phenomenon occurred in only six of the twenty patients studied here, as demonstrated by a slight drop in the caloric consumption as the quantity of amino acids injected was increased. Three of these patients were quite ill. In six other patients the total caloric intake actually increased slightly; that is, the patients requested and received a greater quantity of the high-carbohydrate drink. In the remaining eight patients the caloric intake remained constant throughout the study. In all cases the carbohydrate intake was nearly constant enough so as not to have any influence on the nitrogen requirement. There was therefore no confirmation of the idea that the injection of amino acids had a deleterious effect upon the over-all appetite of the patient, even though immediately after an injection there was a temporary sense of fullness.

Effect Upon Circulating Proteins.—The period of positive nitrogen balance was not extensive enough in these experiments to produce the expectation of a great gain in circulating proteins. The data shown in Table III indicate that though nearly all patients had marked hypoproteinemia there was little change for the better in the quantity of circulating proteins during the study. In fact, in nine subjects there was a significant fall in the quantity of circulating proteins. In the five instances of rise of circulating proteins this rise was associated with an increase in plasma volume rather than an increase in serum protein concentration.

TABLE III. BLOOD PROTEIN STUDIES IN TWENTY PROTEIN-DEFICIENT PATIENTS BEFORE AND AFTER USE OF LYOPHILIZED AMINO ACIDS INTRAVENOUSLY

TABLE III. BLOOD PROTEIN STUDIES IN TWENTY PROTEIN-DEFICIENT PATIENTS BEFORE AND AFTER USE OF LYOPHILIZED AMINO ACIDS INTRAVENOUSLY

PATIENT	TOTAL QUANTITY OF AMINO ACIDS (GM.)	DAYS OF ADMINISTRATION	PLASMA VOLUME (C.C.)	TOTAL SERUM PROTEIN (GM. %)	TOTAL SERUM ALBUMIN (GM. %)	TOTAL CIRCULATING PROTEIN (GM.)	TOTAL CIRCULATING ALBUMIN (GM.)	HEMATOCRIT (%)	WEIGHT CHANGE (KG.)
1	570	10	Before 2,375	5.2	3.6	123.5	85.2	38.0	
			After 1,700	6.5	4.3	111.4	73.0	38.5	−1.8
2	1520	16	Before 2,114	6.1	3.7	128.7	79.5	37.5	
			After 2,710	5.6	4.6	152.8	124.6	39.0	−2.0
3	722	16	Before 3,237	6.3	4.1	203.9	132.4	38.0	
			After 2,983	5.7	3.9	170.5	116.7	35.0	−2.0
4	1606	16	Before 1,925	6.1	3.2	118.2	61.5	45.0	
			After —	4.3	2.7	—	—	34.0	+3.1
5	954	14	Before 3,268	4.2	2.7	140.5	89.8	17.5	
			After 3,000	4.6	2.7	138.9	81.0	19.0	+2.0
6	1606	16	Before 1,604	5.1	3.6	81.9	51.2	35.0	
			After 2,407	5.8	3.6	140.0	86.8	35.0	+2.6
7	1285	17	Before 2,912	5.5	3.4	163.0	98.8	30.0	
			After 2,889	5.1	3.0	150.0	86.7	29.0	−1.9
8	1551	17	Before 3,215	5.8	4.6	189.7	147.8	26.0	
			After 3,424	5.2	3.9	178.1	133.5	26.0	−0.5
9	442	7	Before 2,283	5.1	2.2	116.4	50.3	35.0	
			After 2,193	5.1	2.3	114.0	50.8	35.0	+3.2
10	858	13	Before 2,551	5.8	3.5	148.0	89.2	36.0	
			After 1,927	5.3	3.0	102.0	57.5	32.0	+0.8
11	1668	20	Before 3,096	5.6	2.9	173.7	89.6	40.0	
			After 2,433	6.4	3.5	156.4	85.2	41.0	−2.1
12	1240	17	Before 2,604	5.0	2.2	130.2	57.3	30.0	
			After 2,422	4.6	2.1	111.4	49.8	28.0	+0.8
13	975	15	Before 2,933	6.2	3.7	181.8	108.4	36.0	
			After 2,710	6.3	3.5	173.4	94.7	37.0	+1.0
14	798	16	Before 2,049	7.0	4.1	140.8	84.0	26.0	
			After 1,905	6.7	4.0	127.5	76.2	32.0	−3.2
15	765	14	Before 2,222	6.0	3.3	133.0	73.3	33.0	
			After 1,961	5.9	3.2	116.0	62.8	39.0	+0.9
16	1302	15	Before 2,703	6.3	4.5	170.0	121.6	45.0	
			After 2,857	5.8	3.7	166.0	105.7	39.0	+1.0
17	744	12	Before 2,174	5.9	3.5	127.0	76.1	40.0	
			After 2,900	5.4	3.6	157.0	104.4	32.0	+1.9
18	735	12	Before 1,870	6.0	3.5	113.0	65.5	34.0	
			After 2,400	5.6	3.8	133.0	91.2	35.0	−0.8
19	1014	12	Before 2,381	3.1	1.3	74.0	31.0	35.0	
			After 2,273	3.2	1.4	73.0	31.8	29.0	+1.2
20	194	5	Before 2,526	4.9	2.3	124.0	58.1	35.0	
			After 2,817	4.7	2.4	131.0	67.6	33.0	+1.8

Table III also shows that the patients showed no significant weight change during the period of study. Twelve patients showed gains in weight up to 3 kilograms, but the remaining eight showed losses of equivalent quantities. Both the gains and losses were probably chiefly due to changes in body water content, for in all cases the caloric intake was little more than a maintenance one.

DISCUSSION

The data presented here offer unequivocal evidence that the lyophilized casein hydrolysate used in these studies on patients with chronic protein deficiency is a nutritionally and clinically adequate product. The material can be reconstituted easily with water, glucose solution, or physiologic saline to form a clear solution that is intravenously injectable without reaction. Positive nitrogen balance has been obtained in nineteen of the twenty patients studied. The quantities required for nitrogen equilibrium, though varying from an unusually low value of 42 mg. per kilogram per day to the very high value of 349 mg., averaged 129 milligrams. This means that the average person is likely to obtain his daily nitrogen requirements with an injection of about 60 Gm., or one bottle of these amino acids.

But the adequacy of this protein preparation was determinable in this study by the method of minimal requirements for positive nitrogen balance only by virtue of the large number of balance experiments performed. A random selection of four or five patients might have given values for minimal requirements for equilibrium of the order of 50 mg. per kilogram per day, or they might have averaged 200 milligrams. In the former case the parenterally injected amino acid mixture could have been regarded as superior to the best natural protein mixture given orally. If the latter values had been obtained the product would have been regarded as unsatisfactory. The range was found to be wide in spite of the choice of what was regarded as a homogenous group of patients with chronic protein deficiency and in spite of rigidly controlled regimens with adequate caloric intakes and with the same foods for all patients.

As in Allison's experiments with dogs, the nitrogen intake at equilibrium has been found to be dependent upon the nitrogen excretion on a protein-free diet. If the latter is small, equilibrium is established at a low intake. In general, this means that patients with long-standing protein deficiency require much less nitrogen for equilibrium than patients who have had recent acute losses, as has been found by Browne and co-workers.[11] Yet it was impossible to correlate endogenous nitrogen excretion in these chronically ill patients with the serum protein concentration or total circulating protein. Apparently the exact level of the circulating proteins is determined by many factors rather than merely by the degree of protein deficiency. Apparently, also, there is no easy clinical or laboratory method of anticipating the requirements for equilibrium other than nitrogen balance studies.

The finding of rectilinear curves in the region of negative and low positive balance in thirteen of the nineteen subjects offers proof of the applicability of the concepts of biologic value of proteins as expounded by Mitchell,[12] Allison,[3]

Terroine,[13] and others to the study of a parenterally injected protein product in protein-deficient human subjects. The relative constancy of the K value of these curves—the nitrogen balance index of Allison—in the face of variations in nitrogen intake requirements for equilibrium makes the determination of that value the better gauge of the adequacy of the protein derivative under study. The average value of 0.68 for the nineteen cases, or 0.66 for the more reliable thirteen cases, indicates a good protein. Allison found values of about 0.80 for orally administered casein and of 0.39 for a soybean protein. We have data[14] which indicate that the protein hydrolysate used by us has a much higher biologic value when administered orally. Such a finding is to be expected in view of the fact that the higher plasma amino acid concentrations achieved with intravenous administration promote a greater destruction of amino acids and an increased urinary loss. Thus with oral administration of this product, the K value might approach the value of 0.80.

Yet the use of the nitrogen balance index to characterize a protein, as satisfactory as it is theoretically, is not without its weaknesses in the study of protein-deficient patients. In the first place it requires a long experiment of twenty or more days. In such protracted studies with an unattractive diet and with daily intravenous infusions it is difficult to maintain a constant level of cooperation of the patient and of his emotional reaction toward the dietary management. Besides, his clinical condition and therefore his protein requirements are likely to change during the course of the study. Second, the accuracy of the curve depends primarily upon the establishment of the endogenous nitrogen excretion. This is not easy to determine accurately. When protein is removed from a calorically adequate diet, the nitrogen excretion rapidly diminishes and is usually much lower on the third or fourth day than on the first or second. It has been necessary at times to discard the values of the first and second days. Smith[15] found that the endogenous nitrogen excretion had not become stabilized even after twenty-four days of a nitrogen-poor diet. Third, the slope of the curve depends upon the accuracy of a number of points. The variable errors of the changing condition of the experiment, temperature variations, excretion losses, and analytic defects may combine to produce a succession of points for which no accurate rectilinear curve can be drawn. The number of experiments must be large to avoid the consequences of these inaccuracies.

Though these experiments were not designed for the study of the therapeutic value of parenterally administered amino acids, they offer some corroboration of our previous findings[1, 2] that improvement of patients with long-standing protein deficiency requires an intense and protracted program which is difficult to achieve with parenteral administration alone. Unless the biologic value of hydrolysates is markedly increased by the addition of individual essential amino acids, the quantity of such amino acids that must be administered and the length of the program are too great to be clinically practicable.

The leveling of the curves of nitrogen balance on the high intakes indicates the approach of a ceiling of nitrogen retention. If the retention of each additional increment is no more than 10 or 20 per cent, it becomes doubtful whether

the additional injections are worth the effort and the cost. Undoubtedly, however, there are many patients with severe protein deficiency who must be prepared for operation in as rapid a time as possible at whatever cost. In these instances the intravenous administration of large quantities of amino acids supplemented with whole blood and with as high an oral intake of food as possible might produce the desired results.

SUMMARY

Nitrogen balance studies were carried out in twenty patients with chronic protein deficiency. The experiments were performed with progressive increases in the nitrogen intake, the only significant source of nitrogen being a new parenterally injected lyophilized casein hydrolysate.

The lyophilized product could be injected without reaction in concentrations up to 10 per cent at reasonable speeds for periods up to twenty days.

Positive nitrogen balance was achieved in nineteen of the twenty subjects.

The nitrogen intake required for nitrogen equilibrium varied from 42 to 340 mg. per kilogram per day. The average was 120 mg., with a standard deviation of 51 per cent. The values were directly proportional to the magnitude of the endogenous nitrogenous excretion.

In thirteen of the nineteen cases the curves of nitrogen balance were rectilinear in the region of negative and low positive nitrogen balance; in the remaining six, straight-line curves could be drawn but were not so well defined. In the region of high positive balance the curves tended to level off, indicating a ceiling of utilization. These findings are in corroboration of those of Allison for protein-starved dogs.

Allison's nitrogen balance index (K), which is the slope of the rectilinear curve of nitrogen balance, tended to be constant in all nineteen cases, averaging 0.68, with a standard deviation of 24 per cent. In the thirteen more precise cases, K averaged 0.66, with a standard deviation of 20 per cent. The nitrogen balance index was therefore found to be a more reliable indication of the quality of the protein under study than the nitrogen intake requirement for equilibrium. The latter can be used only when a large number of balance studies are carried out with a homogeneous group of subjects, as in this study. By either criterion the results indicate that the lyophilized casein hydrolysate is the equivalent of a good protein.

The determination of the nitrogen balance index has the following disadvantages: (1) the requirement of a long, tedious, difficult balance study; (2) the inherent error of the estimation of endogenous nitrogen excretion; and (3) the difficulty in drawing an accurate straight-line curve.

Serum protein concentrations or total circulating protein levels were not appreciably increased during the balance studies. A much more intense and protracted regimen is required to alleviate severe chronic protein deficiency.

We wish to express our gratitude to Mrs. Irene Antonow Maxwell who as dietician prepared all menus, and to Miss Lorraine Schmelzle and Mrs. Jacqueline Schaefer for the chemical analyses.

REFERENCES

1. Kozoll, D. D., Hoffman, W. S., and Meyer, K. A.: Nitrogen Balance Studies on Surgical Patients Receiving Amino Acids, Arch. Surg. **51**: 59, 1945.
2. Kozoll, D. D., Hoffman, W. S., Meyer, K. A., and Garvin, T.: High Protein Therapy, Arch. Surg. **53**: 683, 1946.
3. Allison, J. B., and Anderson, J. A.: Relation Between Absorbed Nitrogen, Nitrogen Balance and Biological Value of Proteins in Adult Dogs, J. Nutrition **29**: 413, 1945.
4. Allison, J. B., Anderson, J. A., and Seeley, R. D.: The Determination of the Nitrogen Balance Index in Normal and Hypoproteinemic Dogs, Ann. New York Acad. Sc. **47**: 245, 1946.
5. Hoffman, W. S., and Osgood, B.: The Photometric Microdetermination of Nitrogen Constituents of Blood and Urine by Direct Nesslerization, J. Lab. & Clin. Med. **25**: 856, 1940.
6. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, vol. 2, Baltimore, 1932, Williams & Wilkins Company, p. 78.
7. Gregerson, M. I.: A Practical Method for the Determination of Plasma Volume With the Dye T-1824, J. Lab. & Clin. Med. **29**: 1266, 1944.
8. Wintrobe, M. M., and Landsberg, J. W.: Standardization Technique for Blood Sedimentation Test, Am. J. M. Sc. **189**: 102, 1935.
9. Co Tui, F., Wright, A. M., Mulholland, J. H., Borcham, I., and Breed, E. S.: The Nutritional Care of Cases of Extensive Burns With Special Reference to the Oral Use of Amino Acids (Amigen) in 3 Cases, Ann. Surg. **119**: 815, 1944.
10. Peters, J. P.: Nitrogen Metabolism in Acute and Chronic Disease, Ann. New York Acad. Sc. **47**: 327, 1946.
11. Browne, J. S. L., Schenker, V., and Stevenson, J. A. F.: Some Metabolic Aspects of Damage and Convalescence, J. Clin. Investigation **23**: 932, 1944.
12. Bricker, M., Mitchell, H. H., and Kinsman, G. M.: Protein Requirements of Adult Human Subjects in Terms of Protein Contained in Individual Foods and Food Combinations, J. Nutrition **30**: 269, 1945.
13. Terroine, E. F.: Protein Component in Human Diet, Quart. Bull. Health Organ., League of Nations **5**: 427, 1936.
14. Kozoll, D. D., and Mok, W. T.: Observations on Nitrogen Balance in Surgical Patients With Respect to Caloric Intake and Comparison of Intravenous and Oral Administration of a Casein Hydrolysate. To be published.
15. Smith, M.: The Minimum Endogenous Nitrogen Metabolism, J. Biol. Chem. **68**: 15, 1926.

CYTOCHROME C: INTRAVENOUS ADMINISTRATION IN MAN

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PROGER and Dekaneas[1, 2] have reported the use of cytochrome *c*, intravenously injected, to combat such conditions as myocardial and cerebral anoxia, angina pectoris, acute coronary occlusion, and intermittent claudication. The rationale for using cytochrome *c* in conditions involving tissue anoxia is based on the biochemical role of the pigment. Cytochrome *c* is one of a group of three hemochromogen-like pigments, cytochromes *a*, *b* and *c*, which act as hydrogen transporting agents in tissue oxidations. The *c* compound is soluble, readily extracted from yeast or muscle, and stable. Following the observations of Proger and Dekaneas, supplies of cytochrome *c* have become available from commercial sources for fairly widespread clinical trials.*

Proger and Dekaneas have claimed that successive intravenous injections of 50 mg. cytochrome *c* daily in man may cause this substance to accumulate in the blood stream and form a sort of reservoir upon which the tissues call if subjected to anoxia. The presence of the dark-red pigment was said to have been revealed by a pink discoloration of the serum.

We have administered cytochrome *c* intravenously in doses of 50 to 512 mg. daily for periods up to thirty-one days. Subjects were one normal individual and eight patients with various diseases. The daily and total doses given are shown in Table I. The cytochrome *c* supplied to us was in sterile aqueous solution, 50 mg. per 5 ml. vial. The concentration was determined† using the spectrophotometric method described by Rosenthal and Drabkin.[3] The material was shown by us to be biologically active and of about the stated concentration, as indicated by the increase of oxygen uptake upon addition of different amounts of cytochrome *c* to dilute water homogenate of liver, with addition of calcium and aluminum according to Potter.[4]

Samples of serum and of urine were examined with a hand spectroscope and a rough quantitative estimate of cytochrome *c* was made by comparison of the density of the absorption band of reduced cytochrome *c* (after addition of a little hydrosulfite) with that of known solutions of this substance. Results of these observations are indicated in Table II.

In the one normal adult, injections of 50, 128, 300, and 500 mg. cytochrome *c* intravenously, at intervals of one week, and single doses of 500 mg. administered to two patients suffering from neuromuscular disease brought about no subjective symptoms of ease or of discomfort, and no changes were noted in pulse, respiration, blood pressure, temperature, or basal metabolism despite

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†By John Wyeth & Brother (Canada) Limited, Walkerville, Ontario.

TABLE I. DAILY AND TOTAL DOSES OF CYTOCHROME C

SUBJECT	DAILY DOSE (MG.)	TOTAL DOSE (MG.)
Normal	50-500	978
1 (Als)	50	800
2 (Pma)	512	512
3 (Pma)	100	1200
4 (Mg)	50-100	1400
5 (Als)	50-500	2750
6 (Pma)	50-500	3050
7 (Pma)	100-200	2825
8 (Md)	50-190	4320

Als., Amyotrophic lateral sclerosis; pma., progressive muscular atrophy; mg., myasthenia gravis; md., muscular dystrophy.

almost continuous recordings of these functions for three hours following injection. The cytochrome *c* solution also could be administered intramuscularly without causing undue pain or any reaction.

Total doses of 512 to 4,320 mg. cytochrome *c* injected intravenously in daily doses which varied from 50 to 500 mg. were administered to eight patients suffering from various neuromuscular diseases and to one normal control. These

TABLE II. DETECTABLE CYTOCHROME C

DOSE (MG., I.V.)	SERUM (MIN. AFTER INJECTION)			URINE (HR. AFTER INJECTION)			
	5	30	60	1	3	6	24
50	0	0	0	0	0	0	0
200	0	0	0	+ +	+	0	0
500	+ +	+	0	+ + +	+ +	+	0

injections appeared to be entirely innocuous but produced no detectable changes in symptoms or signs. None of our patients have yet shown any improvement attributable to cytochrome *c*.

From three of the patients and from the normal subject, samples of blood were taken before, shortly after, and twenty-four hours after cytochrome *c* injections. The substance was not detected spectroscopically in the serum of these subjects except where blood samples were collected within thirty minutes after injecting single doses of 500 milligrams. Cytochrome *c* added to serum or water in vitro and reduced with hydrosulfite could not be detected with the hand spectroscope at concentrations of less than about 6.5 mg. per cent. This concentration would correspond approximately to a dose of 325 mg. in a total blood volume of 5 liters. One could not, therefore, expect to detect the cytochrome after smaller injections without more sensitive instruments. A pinkish color of the serum was observed in several samples, but this was found to be due to hemolysis of red blood cells. The bands of oxyhemoglobin were readily seen spectroscopically but no bands of reduced cytochrome *c* could be seen on reduction with hydrosulfite. A solution of cytochrome *c* sufficiently strong to give an obvious pinkish color to serum or urine was found to contain about 20 mg. per cent, a concentration which is readily detectable with the hand spectroscope.

Samples of urine examined within thirty to ninety minutes after injection of doses less than 200 mg. cytochrome *c* did not reveal the presence of this sub-

stance. When the doses injected were from 200 to 500 mg., the samples of urine, passed during the first few hours after injection, ranged from faint rose to deep copper in color, and the characteristic band of reduced cytochrome *c*, ranging from a faint thin line to a broad black band, was visible spectroscopically without the addition of reducing agent.*

CONCLUSIONS

The pinkish color of serum observed by Proger and Dekaneas[1, 2] after injections of cytochrome *c* may have been due to the presence of hemoglobin as a result of slight hemolysis. Cytochrome *c* could be detected in serum by means of a hand spectroscope only immediately after large injections. Considerably greater concentrations of the pigment are required to produce an obvious pink color.

Injected cytochrome is rapidly removed from the plasma and considerable amounts are excreted in the urine.

Intravenous injections of large amounts of cytochrome *c* produce no obvious effects. While harmless, such injections were of no benefit in the few cases of neuromuscular disease studied.

REFERENCES

1. Proger, S., and Dekaneas, D.: Some Effects of Injected Cytochrome *c* in Myocardial Anoxia in Man, J. Pediat. **29**: 729, 1946.
2. Proger, S., and Dekaneas, D.: The Clinical Use of Cytochrome *c* in Patients With Intermittent Claudication, Bull. New England M. Center **8**: 145, 1946.
3. Rosenthal, O., and Drabkin, D. L.: Spectrophotometric Studies; Direct Micro Spectrophotometric Determination of Cytochrome *c*, J. Biol. Chem. **149**: 437, 1943.
4. Potter, V. R.: In Umbreit, W. W., Burris, R. H., and Stauffer, J. F.: Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Minneapolis, 1945, Burgess Publishing Company, p. 95.

*The cytochrome injected was in the oxidized form but the ascorbic acid normally present in the urine would reduce the pigment. Oxidized cytochrome *c*, when added directly to urine, is immediately reduced. If the samples of urine were allowed to remain in open containers, oxidation occurred. The urine turned pale-yellow and the cytochrome *c* band could no longer be detected. However, the rose or copper color and the band invariably reappeared, even in samples which had stood at room temperature for more than three weeks, on dissolving a few crystals of sodium hydrosulfite in the urine.

CARONAMIDE: PLASMA CONCENTRATIONS, URINARY RECOVERIES, AND DOSAGE

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SINCE Caronamide† inhibits the enzyme transport system of the renal tubular epithelium by which penicillin is excreted,[1] the desirability of controlling dosage by accurate measurement of caronamide in the body fluids is obvious. When the initial studies with caronamide were carried out,[2, 3] methods for determining caronamide plasma concentrations were not available. Dosage schedules were established by comparing penicillin dose-response curves modified by caronamide with curves obtained on the same patients when penicillin was administered alone. On this basis it was found that from 9 to 12 Gm. of caronamide per day (1.5 to 2.0 Gm. every four hours) were sufficient to obtain a positive effect in some patients while others required 24 and even 32 Gm. per day (3.0 to 4.0 Gm. every three hours). The observation that different individuals required different dosages to bring about the same effect and the findings of others[4] that age apparently influenced the size of the dose required to give the maximal result, clearly indicated the need for methods for determining caronamide in body fluids in order that dosage could be properly individualized.

It is the purpose of this paper to report the plasma concentrations following various doses of caronamide by the oral and parenteral routes of administration, some recoveries of caronamide in the urine, and the correlation of simultaneously determined caronamide and penicillin plasma concentrations. The results of this work permit a clearer definition of the effective dose of caronamide.

Methods for Caronamide Determinations.—

Method 1: This method,‡ requiring that a forty-eight-hour alkaline dialysate of plasma be analyzed with the Beckman ultraviolet spectrophotometer, proved to be more time consuming and less accurate than Method 3 and was abandoned as soon as the latter procedure became available. It was used, however, during the initial phases of this investigation, and results obtained thereby have been cited previously.[5] Values obtained in this manner show substantial agreement with those obtained by Method 3 since both procedures measure the sum of caronamide and its modified forms.

Method 2: The Brodie method[6] measures only free caronamide and not its conjugates. Acidification of urine or plasma precipitates caronamide which then is dissolved in chloroform. The chloroform solution is extracted with 0.1N sodium hydroxide solution and the resulting alkaline solution of caronamide is read in the Beckman ultraviolet spectrophotometer at 280.5 millimicrons.

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†4′-Carboxyphenylmethanesulfonanilide supplied as Staticin caronamide by Sharp & Dohme, Inc., Glenolden, Pa.

‡This method was worked out by Mr. J. L. Ciminera, Department of Pharmaceutical Chemistry, Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

Method 3: The colorimetric method[7]* depends on the breakdown of caronamide or its modified forms to para-aminobenzoic acid. Diazotization and a coupling reaction, similar to the Bratton-Marshall method for sulfonamide determination, permit accurate estimation of para-aminobenzoic acid. The reliability and simplicity of this procedure recommend it for routine use. Unless otherwise specified, caronamide concentrations referred to below have been determined by this method.

Single Oral Dose of Caronamide.—Penicillin was administered intravenously at a constant rate and penicillin plasma concentrations were determined at half-hour intervals during a control period and then at half-hour intervals after the oral administration of caronamide. These observations revealed that 4 Gm. of caronamide elevated the penicillin plasma concentration as early as

Caronamide Plasma Concentrations following a Single Oral Dose (4.5 Gm.) of Caronamide

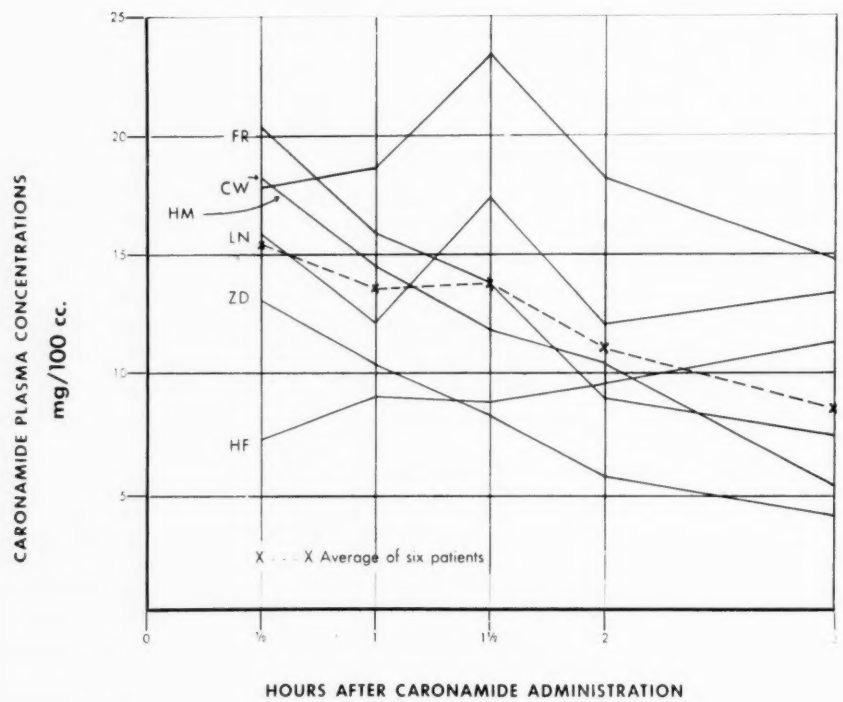

Fig. 1.

thirty minutes after its administration and the effect of the drug persisted for from five to seven hours. Methods for determining caronamide in the plasma were not available at that time and the concentrations of the compound that produced these results, therefore, are not known.

When methods became available, six patients were given 4.5 Gm. of caronamide in a single oral dose, the drug being administered as the sodium salt in a 7.5 per cent solution. Fig. 1 presents the individual caronamide dose-

*In the reference cited this method is referred to as procedure III, Alternative Determination in Plasma.

response curves and it is observed that, even in this small group, there were marked differences in handling of the drug. The average values obtained from estimation of caronamide plasma concentrations of the six patients were 15.5 mg. per 100 c.c. after one-half hour and 8.0 mg. per 100 c.c. of plasma at the end of three hours.

Repeated Oral Doses of Caronamide.—Six patients, chosen at random in order that they might better represent the clinical conditions under which caronamide is likely to be administered, were given 3 Gm. of the drug at three-hour intervals for five doses. One hour after all but the first dose a blood specimen was drawn for caronamide determination. From the averaged results (Fig. 2) it is clear that over the period of study there was a gradual rise in caronamide plasma concentration from 6.5 to 18.5 mg. per 100 c.c. of plasma. All of these patients tolerated the drug well and no untoward symptoms were observed.

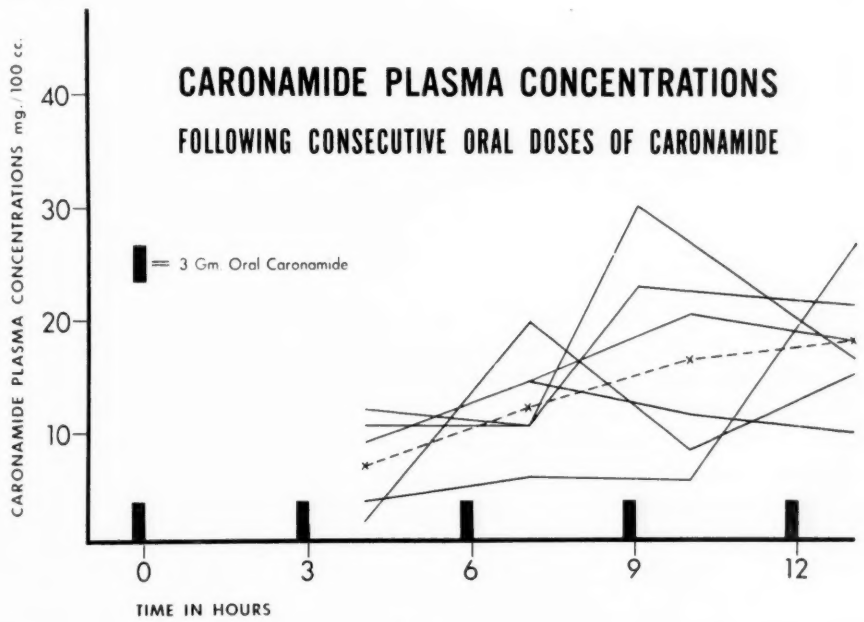

Fig. 2.

Since there appeared to be some tendency for the plasma concentration to rise gradually during a twelve-hour period following initial administration of caronamide, a similar study was conducted but observations were extended for a longer time. Five patients were given 1.5 Gm. of caronamide every three hours and five patients received 3.0 Gm. every three hours for one week. Several patients left the hospital before completion of the study and did not receive the drug for the full week. A plasma caronamide determination was made daily, the blood specimen being drawn at approximately the same time each day from one to two hours following a dose of caronamide. Determinations on the plasma of Patient L. N. were estimated by Method 1; all the other results presented

CARONAMIDE PLASMA CONCENTRATIONS* FOLLOWING CONTINUOUS ORAL ADMINISTRATIONS

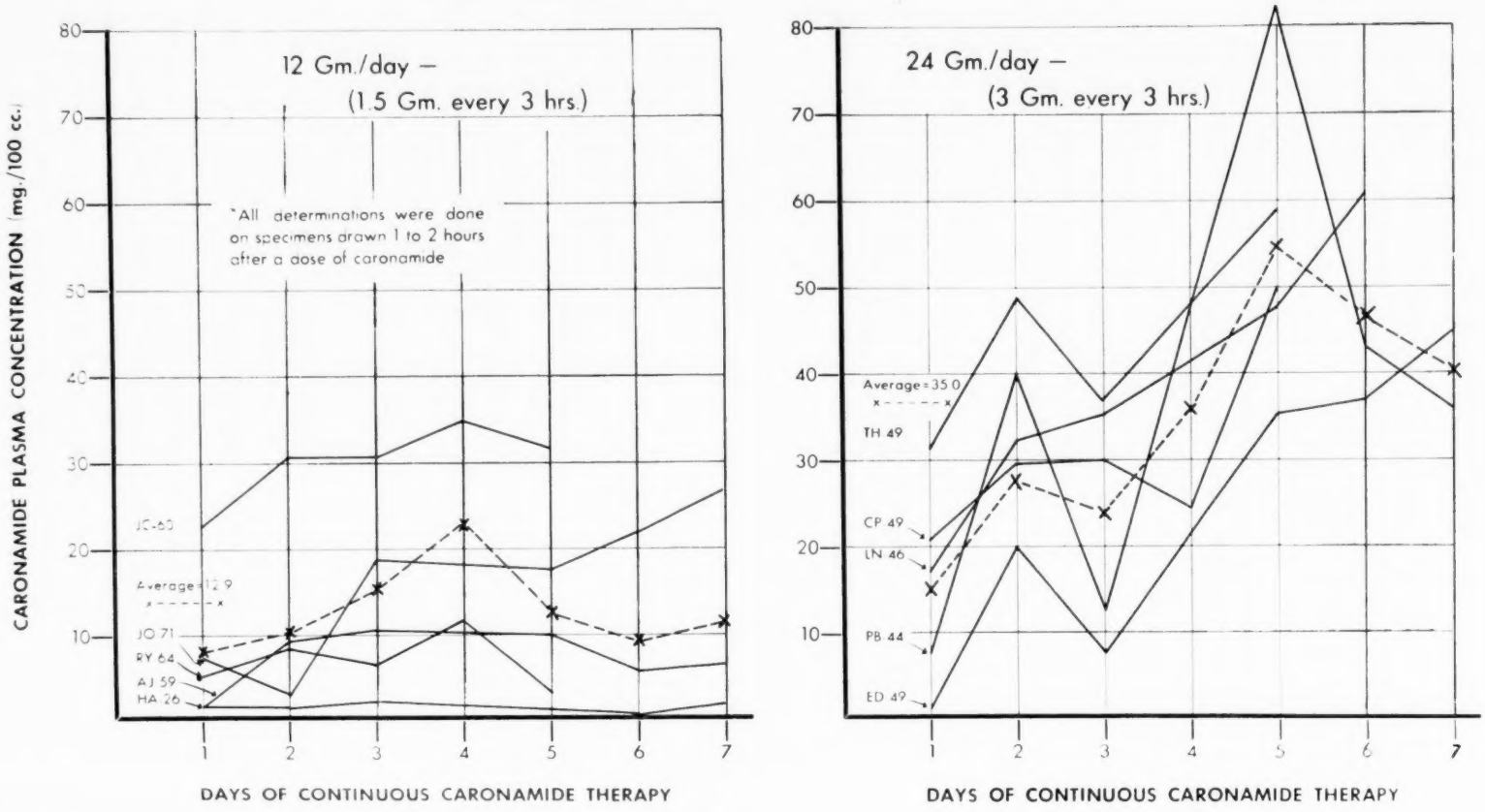

Fig. 3.

in Fig. 3 were obtained by Method 3. Since Methods 1 and 3 both measure caronamide and its modified forms, however, the results are considered together.

Despite marked individual differences in the handling of the drug, the difference between the plasma concentrations of patients on the two dosage schedules is clearly defined. The averaged data on the patients who received 3 Gm. of caronamide every three hours show a rising concentration of the compound. As noted in Fig. 3 the caronamide determinations were done on blood specimens drawn either one or two hours after a dose of caronamide. From dose-response curves following a single dose of caronamide, it has been observed that the one-hour determinations are uniformly higher than those done two hours after medication. In consequence, some of the tendency of the average figures in Fig. 3 to rise over the seven-day period may be more apparent than real. That this is probable is confirmed by observations that during more prolonged administration of caronamide increasing plasma concentrations did not occur.

Since Intravenous Dose of Caronamide.—Although one of the chief advantages of caronamide is the fact that it is administered orally, some experiments with intravenous caronamide have been done.

Three patients were given a 7.5 per cent solution of sodium caronamide intravenously, 3 Gm. being injected over a period varying from three to seven minutes. All of these patients experienced a mild sensation of warmth after 15 to 20 c.c. of the solution had been injected, but otherwise there were no reactions. At the same time 200,000 units of penicillin were injected intravenously in order that it might be determined whether or not the plasma concentrations of caronamide were sufficient to produce an elevation of the penicillin plasma concentrations as compared with those obtained in a control period without caronamide. In all instances a marked elevation of the penicillin concentration was noted (particulars of these experiments will be reported in another publication), indicating that the concentrations of caronamide (Table I) were effective in inhibiting penicillin excretion. Observations were limited to an hour and forty minutes and during this period quantitative urine collections were made, making possible the measurement of caronamide in the urine (Table I).

TABLE I. CARONAMIDE PLASMA CONCENTRATIONS AND URINARY RECOVERIES FOLLOWING A SINGLE INTRAVENOUS DOSE OF CARONAMIDE

PATIENT	AGE	I.V. DOSE OF CARONAMIDE (GM.)	PLASMA CONCENTRATIONS OF CARONAMIDE* (MG./100 C.C.)				RECOVERY IN URINE*	
			40 MIN.	60 MIN.	80 MIN.	100 MIN.	(GM.)	(%)
D. S.	41	3	10.07	8.75	7.20	6.27	1.15	38
K. S.	34	3	12.30	9.62	8.24	6.25	1.45	48
J. H.	38	3	15.15	10.90	7.56	6.70	.66	21.9
						Average	1.08	35.9

*All determinations done by Method 2 (Brodie[6]).

Continuous Intravenous Infusion of Caronamide.—Three patients in apparent good health and with normal renal function received 3 Gm. of sodium caronamide intravenously as a priming dose (injection time, three to five

minutes) and thereafter were infused with a solution containing sodium caronamide (Table II). The actual amounts of caronamide administered were 5.04, 4.95, and 4.3 Gm., respectively, to Patients I. S., M. F., and B. B. The caronamide plasma concentrations that were noted following these doses are given in Table II. These determinations were made by employing Method 2.

TABLE II. CARONAMIDE PLASMA CONCENTRATIONS FOLLOWING CONTINUOUS INTRAVENOUS ADMINISTRATION OF CARONAMIDE

PATIENT	AGE	CARONAMIDE		CARONAMIDE PLASMA CONCENTRATIONS* (MG./100 C.C.)			
		PRIMING DOSE (GM.)	INFUSION (MG./KG./HR.)	45 MIN.	60 MIN.	75 MIN.	90 MIN.
I. S.	44	3	23.1	25.8	26.63	23.40	22.65
M. F.	17	3	32.3	24.73	23.44	18.02	25.42
B. B.	39	3	23.2	20.60	18.81	19.18	18.30

*All determinations done by Method 2 (Brodie[6]).

Recoveries of Caronamide After Oral and Intravenous Administration.—Five patients (five of the patients listed in Fig. 3) were given caronamide every three hours for five days and on the sixth day, while the drug was still being administered, twenty-four hour urine specimens were collected. The total amount of caronamide in this collection was determined both gravimetrically and by Method 3. For the gravimetric determination caronamide is precipitated by acidifying the urine to a pH below 5.0 and thereafter the precipitate is washed, dried, and weighed. By this method only caronamide is measured, since its modified forms remain in solution. Method 3 measured the modified forms of caronamide in solution and hence the values obtained are higher than those representing only caronamide (Table III).

TABLE III. RECOVERIES OF CARONAMIDE FROM TWENTY-FOUR HOUR URINE COLLECTIONS

PATIENT	AGE	CARONAMIDE (GM./24 HR.)	24-HR. URINE VOL. (C.C.)	CARONAMIDE IN URINE		RECOVERIES (%)	
				(GM./100 C.C.)*	TOTAL*	GRAVIMETRIC	COLORIMETRIC
R. O.	71	12	1490	0.1245	1.805	15.04	42.0
H. A.	26	12	1275	0.7153	9.020	75.1	75.3
P. B.	44	24	1300	0.3754	5.632	23.4	43.0
L. N.	46	24	2300	0.5071	11.853	49.3	51.0
E. D.	49	24	1390	0.2485	3.404	14.1	25.0
					Average	35.38	47.26

*All values represent the average of duplicate aliquots.

Correlation of Caronamide Plasma Concentrations With Penicillin Plasma Concentrations.—Dosage schedules for caronamide originally were set up without the benefit of caronamide plasma concentrations by determining the dose of caronamide that influenced favorably plasma concentrations of penicillin. When methods became available for determining caronamide in body fluids, the dosage schedules previously established were re-evaluated.

Penicillin dose-response curves modified by caronamide were compared with penicillin dose-response curves obtained on the same patients when penicillin alone was administered. For each curve the dose of penicillin was the same and, when the curve modified by caronamide showed at least a twofold

CORRELATION OF CARONAMIDE AND PENICILLIN PLASMA CONCENTRATIONS

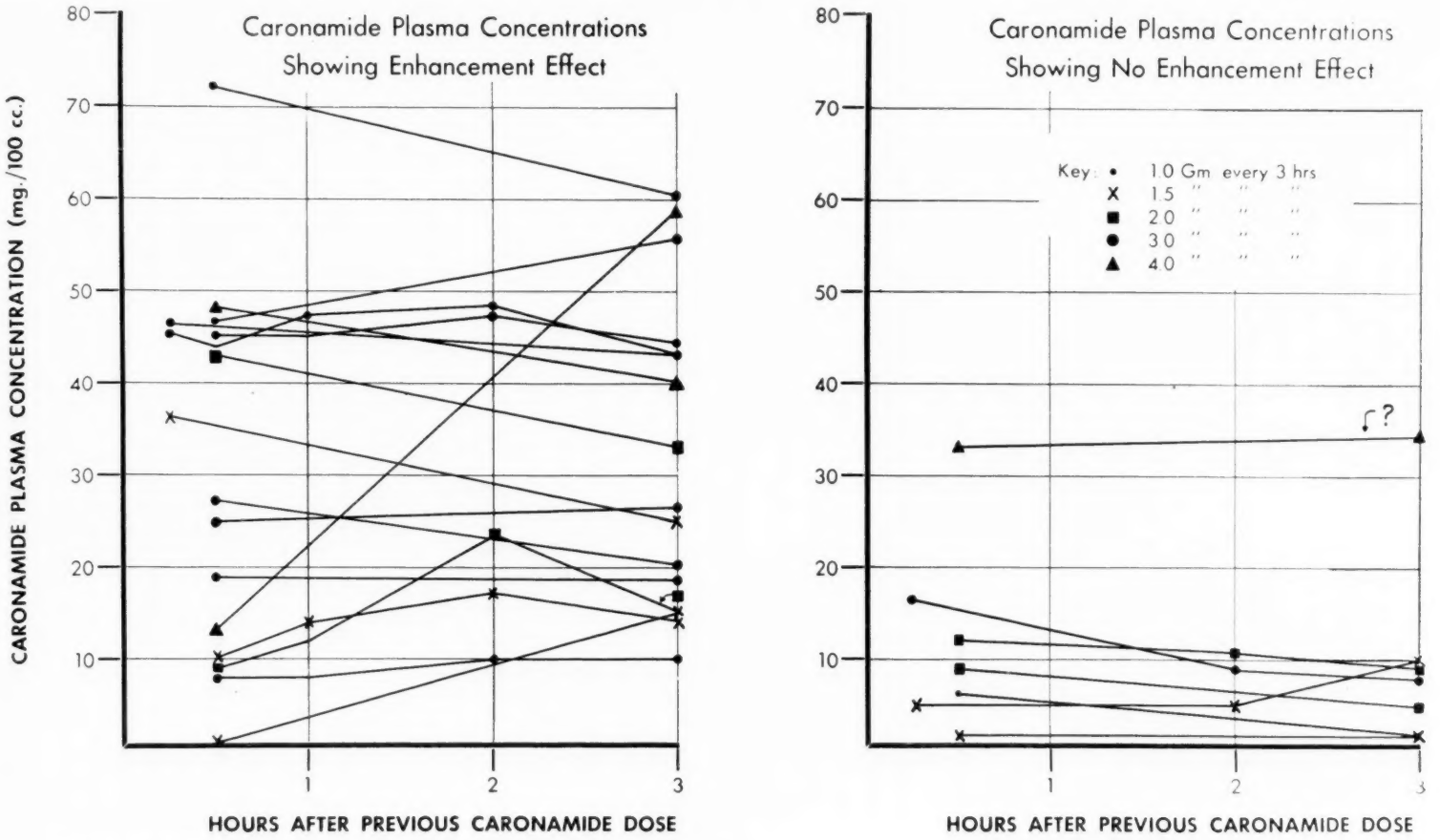

Fig. 4.—(Boger, W. P.: Caronamide: A New Enhancing Agent for Use in Conjunction With Penicillin Therapy. Tr. & Stud., Coll. Physicians, Philadelphia 15: 104, 1947.)

enhancement over the control curve, the result was regarded as satisfactory. On this basis, caronamide plasma concentrations were correlated with penicillin plasma concentrations (Fig. 4).

From Fig. 4 it is apparent that a caronamide plasma concentration of 15 mg. per 100 c.c. approximates the critical value that determines the inhibition of the excretion of penicillin. The striking exception presented has been questioned because there was very real doubt that the patient received the prescribed amount of penicillin. The criteria used to determine the positive and negative effects of caronamide demand that the patient receive the same dose of penicillin in the control period and the caronamide treatment period. If this requirement is not fulfilled, a failure of caronamide may be implied where none exists.

DISCUSSION

It has not yet been determined whether or not the metabolic products of caronamide are physiologically active. For this reason there would appear to be some advantage in using a method for determining caronamide that measures both the drug itself and its conjugates. Methods 1 and 3 fulfill this requirement, but because of its simplicity Method 3 is preferred.

Method 2 measures caronamide alone and, when aliquots of the same specimen have been analyzed by Methods 2 and 3, the values of Method 2 have been roughly half of those obtained by Method 3. Despite this relationship it would be hazardous to depend on it, for not all patients conjugate caronamide to the same degree. The urinary recoveries of caronamide in only a small group of patients (Table III) showed that patients such as H. A. and L. N. either do not conjugate the drug at all or do so to only a slight degree. Other patients excreted almost half the caronamide as a conjugate or metabolic product of caronamide. It is apparent, therefore, that values obtained by Method 2 will not bear a constant relationship to values obtained by Method 3. Application of the two methods in the study of a larger series of patients should give valuable information concerning the metabolism of caronamide.

It has been noted previously[8] that a reducing substance occasionally appears in the urine following caronamide administration. Nine of the ten patients presented in Fig. 3 showed this reducing substance in the urine. The exact nature of this substance has not been determined, but it reduces Benedict's solution, is still present in specimens subjected to yeasting, and gives a positive Bial's test. The last mentioned test generally is performed to determine the presence of a pentose. It is interesting to note that a reducing substance has been observed in the urine of persons treated with para-aminobenzoic acid[9] and it is not unlikely that this reducing substance is the same as that observed following the administration of caronamide.

The demonstration that caronamide is effective in elevating penicillin plasma concentrations from two to seven times led to the conjectures whether the intervals between intramuscular injections of penicillin could be lengthened and whether caronamide might prolong the period of activity of a single intramuscular dose of penicillin in aqueous solution to equal the duration of action

of an injection of penicillin in oil and beeswax. The duration of action of a single dose of caronamide and the plasma concentration of caronamide required to inhibit penicillin excretion are intimately related to these questions.

At the beginning of investigations with caronamide, clinical evaluation was handicapped by the inability to determine the compound in the plasma. Nevertheless, it was found that a single 4 Gm. dose of caronamide exerted an effect on penicillin plasma concentration for from five to seven hours. Later, when methods for caronamide assay were developed, it was found that a slightly larger single dose, 4.5 Gm., provided plasma concentrations between 8 and 15 mg. per 100 c.c. (Method 1) for a period of three hours.

These plasma concentrations in man are in good agreement with the theoretic figure of 6 mg. per 100 c.c. that is regarded as giving a good suppressive effect upon penicillin excretion in dogs.[10] Further, the finding that the oral administration of a single 4 Gm. dose of caronamide, that is, approximately 50 mg. per kilogram of body weight for an average adult weighing 70 kilograms (154 pounds), elevated plasma penicillin concentrations for from five to seven hours, confirms the finding in dogs that a single oral dose of 50 mg. per kilogram, that is, 1 Gm. for a dog weighing 20 kilograms, decreased penicillin clearance for at least four hours.[10]

Since 4 Gm. of caronamide as a single oral dose elevated penicillin plasma concentrations for from five to seven hours and a slightly larger dose (4.5 Gm.) of sodium caronamide given orally resulted in a plasma concentration of 8 mg. per 100 c.c. (Method 2) for only three hours, it is suggested that even lower concentrations can partially inhibit penicillin excretion.

Reasoning from the foregoing, a dose of 4 Gm. every four hours would maintain a plasma concentration of caronamide close to the one required definitely to inhibit penicillin excretion. Using this schedule of dosage it has already been shown[4] that 100,000 units of penicillin in aqueous solution will sustain assayable penicillin levels in the plasma for as long as eight hours. It is doubtful whether caronamide doses can be spaced farther apart than every four hours since it is excreted rather rapidly, and it seems desirable to maintain an adequate caronamide plasma concentration in order to assure at least a twofold increase in the level of penicillin.

It has not yet been determined whether by continued administration of caronamide it will be possible to prolong the effects of a single injection of aqueous penicillin to equal that of penicillin in oil and beeswax. However, if assayable levels can be obtained for as long as eight hours after 100,000 units of aqueous penicillin when caronamide is given, the likelihood is great that, with larger doses of penicillin, assayable levels can be extended to at least twelve hours. To prolong the effect of a single injection of penicillin and to circumvent the necessity of relying upon the patient to take additional medication, single oral doses of 6 Gm. of caronamide have been given in conjunction with 500,000 units of oral penicillin. This treatment has been applied to a group of patients with acute gonorrhea and the rate of cure has been 87 per cent.[11] Caronamide plasma concentrations following the administration of 6

Gm. are not available at this time but, in consideration of the results obtained following a 4 Gm. dose, it might be anticipated that 6 Gm. of caronamide would produce a concentration that would influence penicillin plasma levels for at least six to ten hours. Apart from the inconvenience of swallowing the tablets, 6 Gm. doses have been given without unfavorable reactions and investigation of large single doses of caronamide is indicated.

In Fig. 3 the impression is given that a fixed dose of 3 Gm. every three hours may cause an accumulation of the drug in the plasma. That this rising concentration may be more apparent than real has already been mentioned. It is worth while to point out, however, that we have had the opportunity of observing other patients not reported here who have taken 3 Gm. every three hours for two and three weeks without showing either increasing plasma concentrations or symptoms of systemic toxicity. An occasional patient has complained of nausea and a few have vomited. It has been difficult to differentiate between simple nausea induced by the difficulty incident to swallowing a number of large tablets and that caused by drug toxicity. It has been interesting to observe that a number of these patients have been able to continue medication at the same dosage level when a suspension of caronamide replaced the tablets. However, several instances of vomiting probably due to drug toxicity have been encountered, and in two of these patients it has been shown that caronamide plasma concentrations were in excess of 70 mg. per 100 cubic centimeters. This finding only emphasizes the desirability of controlling dosage by caronamide plasma determinations. It should be pointed out that caronamide concentrations of 45 mg. per 100 c.c. and above generally are well tolerated (Figs. 3 and 4).

In a previous publication,[5] on the basis of only two patients, it was suggested that 20 to 30 mg. per 100 c.c. (Method 1) was the caronamide plasma concentration required to suppress the elimination of penicillin. On the basis of a larger experience and more reliable caronamide determinations, it appears that concentrations as low as 8 mg. per 100 c.c. (Method 2) partially inhibit penicillin excretion and concentrations between 10 and 20 mg. per 100 c.c. insure at least a twofold enhancement of penicillin plasma concentration (Fig. 4). Concentrations between 20 and 40 mg. per 100 c.c. are well tolerated and they approximate the plasma concentration of caronamide that provides optimal inhibition of penicillin excretion.

The dose that has most frequently fulfilled the requirement of maintaining for the entire time between doses of caronamide a plasma concentration that is adequate to inhibit penicillin excretion has been 3 Gm. every three hours or 4 Gm. every four hours. In a few instances this dose has failed to maintain the desired plasma concentrations and in others (Fig. 3, Patients J. C. and J. O.; and Fig. 4) 1.5 Gm. every three hours have maintained the requisite level. Actually, the optimal dose of caronamide cannot be defined arbitrarily as a certain number of grams per day; the dosage necessary to maintain the plasma concentration at or above 15 mg. per 100 c.c. varies from individual to individual and varies somewhat even in the same individual from day to day.

Diet, degree of hydration, fluid intake, fever, and disease influence to a marked degree the daily requirements of any particular patient. The avail-

ability of a simple and reliable method for caronamide determination has established this clearly. Striking differences in individual requirements under conditions other than those just mentioned are probably a reflection of the patient's renal function. It appears that the more nearly normal renal function is, the larger the dose of caronamide required to maintain a plasma concentration that will inhibit penicillin excretion. This generalization is compatible with the available clinical data. Children with normal renal function have been found to require daily doses equal to adult doses, 24 Gm. per day,[12] in order to obtain elevations of penicillin plasma concentration. Persons over 60 who, presumably on the basis of aging alone, have some renal impairment require less caronamide to inhibit penicillin excretion than do younger individuals.[4] Caronamide probably is excreted only by glomerular filtration and, consequently, impairment of glomerular function will retain the drug in the circulation, thus making smaller doses more effective. Furthermore, since the site of action of caronamide is the renal tubule, any existing impairment of tubular function means that less drug will inhibit those tubules that are functioning normally. Thus if a patient to whom caronamide is administered has subclinical renal impairment, a relatively small dose of the drug will be required to maintain an optimal plasma concentration.

CONCLUSIONS

A caronamide plasma concentration of approximately 15 mg. per 100 c.c. is required to inhibit the renal excretion of penicillin sufficiently to provide at least a twofold elevation of penicillin plasma concentration. A single oral dose of 4 Gm. of caronamide will not maintain this concentration in the plasma but, by reason of partial inhibition of the renal tubules, will, nevertheless, influence penicillin excretion for from five to seven hours. Although 1.5 Gm. every three hours in some patients provide this critical level (15 mg. per 100 c.c.) in the plasma, 3 Gm. every three hours or 4 Gm. every four hours are required in the majority of patients with normal renal function. Caronamide plasma concentrations of 20 to 40 mg. per 100 c.c. are well tolerated and probably represent the concentrations that maximally inhibit the tubular excretion of penicillin. There are marked individual differences in the metabolism of this compound, but the average twenty-four hour recovery of free caronamide is 35.38 per cent and of caronamide and its metabolic products, 47.26 per cent of the material ingested. The availability of a simple and reliable method for caronamide determination permits individualization of caronamide dosage.

We wish to acknowledge our indebtedness to Dr. Harrison F. Flippin and Dr. William G. Leaman, Jr., for having granted permission to study patients on their services at the Philadelphia General Hospital, Philadelphia, Pa.

REFERENCES

1. Beyer, K. H.: New Concept of Competitive Inhibition of the Renal Tubular Excretion of Penicillin, Science **105**: 94, 1947.
2. Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K.: Caronamide for Increasing Penicillin Plasma Concentrations in Man, J. A. M. A. **134**: 1528, 1947.

3. Shaw, C. C., Boger, W. P., Crosson, J. W., Kemp, W. W., Ling, W. S. M., and Duncan, G. G.: Enhancement of Penicillin Blood Levels in Man by Means of a New Compound, Caronamide, Am. J. Med. **3**: 206, 1947.
4. Seeler, A. O., Wilcox, C., and Finland, M.: Enhancement of Blood Levels by Caronamide During Intramuscular Administration of Penicillin, J. Lab. & Clin. Med. **32**: 807, 1947.
5. Boger, W. P., Kay, C. F., Eisman, S. H., and Yeoman, E. E.: Caronamide, A Compound That Inhibits Penicillin Excretion by the Renal Tubules, Applied to the Treatment of Subacute Bacterial Endocarditis, Am. J. M. Sc. **214**: 493, 1947.
6. Brodie, B. B., Levy, B., and Bernstein, E.: The Estimation of Caronamide (4′-Carboxyphenylmethanesulfonanilide) in Biological Fluids, J. Pharmacol. & Exper. Therap. **91**: 246, 1947.
7. Ziegler, C., and Sprague, J. M.: A Colorimetric Determination of Caronamide, J. Lab. & Clin. Med. **33**: 96, 1948.
8. Rapoport, M., Corneal, F. B., Beyer, K. H., and Verwey, W. F.: Clinical Evaluation in Children of the Toxicity and Efficacy of Caronamide for the Competitive Inhibition of Penicillin Excretion, Proc. Soc. Pediat. Res. page 7, 1947.
9. Snyder, J. C., Yeomans, A., Clement, D. H., Murray, E. S., Zarafonetis, C. J. C., and Tierney, N. A.: Further Observations on the Treatment of Typhus Fever With Para-Aminobenzoic Acid, Ann. Int. Med. **27**: 1, 1947.
10. Beyer, K. H., Miller, A. K., Russo, H. R., Patch, E. A., and Verwey, W. F.: The Inhibitory Effect of Caronamide on the Renal Elimination of Penicillin, Am. J. Physiol. **149**: 355, 1947.
11. Vest, Samuel: Personal communication.
12. Corneal, F. B., and McNair Scott, T. S.: Personal communication.

ISOLATION AND IDENTIFICATION OF INFLUENZA VIRUSES DURING THE EPIDEMIC OF DECEMBER, 1945

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THE ease with which influenza viruses can be isolated and identified from individuals ill with clinical influenza has varied considerably in different epidemics, in different laboratories during the same epidemic, and even in different patients studied in the same laboratory during the same outbreak. During the epidemic of influenza A which occurred in Boston in December, 1943, and in sporadic cases that occurred during the ensuing weeks, successful virus isolations were made quite readily from the throat washings of a small group of patients with clinical influenza and from the lungs of patients with fatal cases.[1] These isolations were accomplished by using unfiltered and untreated throat washings or lung suspensions either for intranasal mouse inoculation and passage or for inoculation and subsequent passage through the allantoic sac of developing chick embryos or by combinations of these methods. Bacterial contaminations were usually eliminated in the course of the mouse passages without resort to special methods, or by filtration after the virus had multiplied and become established either in the mouse lungs or in the allantoic fluid of the chick embryo. Similar methods were used successfully during the same epidemic by workers in Minnesota.[2, 3]

The same methods, except for the addition of adequate amounts of penicillin to suppress bacterial contaminations, were entirely without success when used on materials collected from patients during and after the epidemic of influenza B that occurred in and around Boston in December, 1945. After numerous attempts, influenza B viruses were eventually isolated from throat washings of a number of patients studied during this epidemic, utilizing other routes of inoculation of embryonated eggs both with and without preliminary mouse passage.† In view of the difficulties encountered and the different methods employed, it may be of interest to present here some of the details of the experiences with the isolation of influenza viruses during this outbreak. The results have been reported elsewhere of the serologic studies made in cases and family contacts from a localized outbreak in a school in one of Boston's suburbs,[4] in cases of uncomplicated respiratory infections,[5] and in patients with pneumonia[6] seen at the Boston City Hospital during this epidemic.

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†Only three strains had been successfully established at the time that the report was made on the serologic studies in cases and family contacts from the school outbreak in Needham, and only one of those strains was from a Needham case.[4] Several other strains were subsequently isolated on one or more attempts both from children in Needham and from patients at the Boston City Hospital, as detailed in this paper.

MATERIALS AND METHODS

Throat Washings.—These were taken on the first to the third day of the disease and only from patients who at the time were acutely ill with a fever of 101° F. or higher and with clinically typical influenza. The patient, after first rinsing the mouth with tap water, gargled two or three times with 20 ml. of brain-heart infusion broth containing 10 per cent horse serum. The garglings were then distributed in Pyrex tubes which were sealed and stored in a carbon dioxide icebox until used. Whenever feasible, however, the fresh washings were first inoculated directly into eggs and mice. For the inoculations into eggs, enough crystalline penicillin G was added to the inoculum to give a final concentration of 1,500 to 5,000 units per milliliter. Untreated washings were used for the mouse inoculations.

Virus Isolations.—Embryonated hens' eggs and mice were used for virus isolations. The former were each inoculated by one of three routes—amniotic, embryonic, or allantoic.

Amniotic Inoculations.—This was done by a modification of the method of Burnet,[7] using six eggs each time whenever possible. Nine-day embryonated eggs were placed, with the air sacs up, over an egg candler containing a very bright light. The eggs were rotated until the embryos were visualized and a small opening was made with a dental drill in the shell over the air sac. The inoculation was then made through this hole directly into the amniotic sac with a 22 gauge needle one and one-half inches long. When this was properly done, the force of the injected fluid moved the embryo away from the point of the needle. The inoculation of dyes in this manner has usually shown the fluid to be contained within the amniotic sac. After the inoculation, the holes were sealed with collodion to which some half-strength tincture of iodine was added. The eggs were then incubated for four days at 35° C. when influenza B viruses were anticipated, or at 37° C. for influenza A viruses.

After incubation the eggs were chilled for one hour in a deep-freeze refrigerator at about −20° C. to prevent bleeding. The shell over the air sac was then removed with forceps and the membranes carefully torn away. Both amniotic and allantoic fluids were usually collected and tested separately for the presence of virus. This was done by adding 0.5 ml. of a 1.5 per cent suspension of adult hen cells to tubes each containing a similar volume of serial twofold dilutions of the amniotic or allantoic fluids in saline and allowing the mixtures to stand at room temperature for one hour. The tubes were then read for hemagglutination.[8] In almost every case in which successful isolation of virus was accomplished, the presence of virus was detected in this manner in the amniotic fluid of the first egg passage. Further blind amniotic passages were made, usually up to five times, before an attempt was discarded as a failure.

Embryonic Inoculations.—In this method,[9] thirteen-day chick embryos were used and an inoculum of 0.2 ml. was injected directly into the embryo itself rather than into the amniotic sac. Six eggs were generally used for early passages. After incubation for forty-eight hours the allantoic fluid was removed and tested for agglutination with chicken red cells. The tracheae were then

dissected out, pooled, ground with sterile alundum, and made up to a 10 to 20 per cent suspension with broth containing 10 per cent normal horse serum. This suspension was used for further egg passages when the allantoic fluid failed to agglutinate the chicken cells.

Allantoic Inoculations.—For this purpose 10- to 11-day-old embryonated eggs in groups of four to six were inoculated directly into the allantoic sac in the manner described by Hirst.[8] A hole was drilled on the side of the egg over the embryo but in an area where there were no large blood vessels. A second puncture was made in the shell over the air sac. An inoculum of 0.1 ml. was then injected with a 26 gauge needle one-half inch long through the side opening directly into the allantoic sac. Both holes were then sealed with the collodion-iodine mixture. After two days of incubation at 35 or 37° C., depending on whether influenza B or A virus, respectively, was anticipated, the allantoic fluid was harvested without previous chilling. The large blood vessels in the chorioallantois were ruptured and allowed to bleed into the allantoic cavity. The allantoic fluid containing the embryonic red cells was collected, pooled, and concentrated according to the method of Francis and Salk.[10]

Mouse Inoculations.—Mice in groups of six, each weighing 10 to 12 grams, were inoculated intranasally under light ether anesthesia with 0.05 ml. of untreated throat washing. The mice were sacrificed on the third day, the lungs dissected out, and the presence or absence of lesions was noted. The lungs were then pooled, ground with sterile alundum, and made up to approximately a 20 per cent suspension in 10 per cent horse serum broth. After slow centrifugation to remove coarse particles, the supernatant fluid was removed and used for further passages.

Controls.—Sterile horse serum broth and negative or unknown materials, including throat washings and lungs from patients without clinical influenza, were carried through all of the various egg and mouse procedures and yielded negative results in every instance. Care was taken not to work with established viruses on days when isolation procedures were being carried out.

Serologic Tests.—The methods used for titrating the viruses and for the agglutination inhibition and complement fixation tests were similar to those used in previous studies reported from this epidemic.[4] Neutralization tests were carried out in chick embryos by the method of Hirst.[11] The PR8 and Lee strains* have been maintained by allantoic passages in eggs. The BON strain[12]† was not introduced into the laboratory until most of the strains from this epidemic had already been isolated.

Identification and differentiation of the viruses were carried out, in part, by the use of antisera prepared in rabbits. For this purpose albino rabbits weighing 2.5 to 3.5 kilograms were used, two for each strain of virus. Each rabbit was bled about 10 ml. from the marginal ear vein to obtain serum for controls in subsequent tests and was then injected intravenously with 0.1 ml. of concentrated active virus. The rabbits were again bled from the ear vein after ten to fourteen days, and the serum obtained at this time together with

*Originally obtained from Dr. Thomas Francis, Jr., School of Public Health, University of Michigan.

†Obtained from Dr. John F. Enders, Department of Bacteriology, Harvard Medical School.

the controls was heated at 60° C. for twenty minutes and tested for inhibition of chicken cell agglutination by the homologous virus. If there was a satisfactory rise in titer, the rabbits were bled by cardiac puncture and the serum collected and stored in sealed tubes at –20° C.

RESULTS

Virus Isolations.—Only fourteen throat washings obtained from patients with clinically typical cases of influenza were studied. Each of these washings, however, was used in from two to twenty-seven separate attempts to obtain virus by inoculation and passage, utilizing various methods or combinations of methods. The source of the washings, the antibody titers of influenza A (PR8) and influenza B (Lee) in the serum of the patients from whom they were obtained, and the results of the various attempts to demonstrate the presence of virus in these washings are summarized in Table I.

Successful isolations were made on one or more attempts from eight of these fourteen throat washings. All of these eight washings were obtained between December 13 and 21 from patients who developed significant rises in titer of antibodies for influenza B and not for influenza A.* The six which failed to yield any virus include all three of the washings that were obtained after the middle of January from patients who developed a rise in antibodies for the PR8 strain of influenza A and not for the Lee strain nor for any of the recently isolated strains of influenza B. Interestingly enough, two of the other three failures were with the latest washings obtained from patients with serologic evidence of influenza B. The serums of two of these patients, W. D. and J. C., showed, in addition, fourfold or greater rises in antibodies to three strains (MB, MF, and WC) of influenza B that were isolated from this epidemic. The washings from J. C., however, were not carried through the usual blind passages in eggs. Of all the six washings which failed to yield a virus, only the one from W. D. was obtained on the third day of illness; the others were collected on the first or second day.

With the washings that yielded viruses, the same methods were not always equally successful. There were not enough observations made with each method or combination of methods to permit definite comparisons. The available data, nevertheless, seem to warrant some deductions as to the relative efficacy of the different methods. The amniotic route of inoculation was definitely the most successful for the direct isolation of the viruses in eggs. In no instance could virus be established if washings were inoculated initially by the allantoic route. Inoculation directly into the embryo was not used as often as the other methods and was somewhat more cumbersome, but it seemed to yield results similar to those obtained by amniotic sac inoculations.

Mouse inoculation and passage was not successful alone in establishing any of the viruses. Indeed, when typical lesions were present in the lungs of mice inoculated with the original washings, the lesions usually became progressively

*Since this paper was submitted successful isolations of influenza B virus were made from stored throat washings of Case 10 (J. C.) on three separate occasions. The virus was first recognized in Am_1 in one instance, in M_1Al_2 in a second, and in M_1Am_2 in the third. The first was maintained through Am_1Al_2, the second through M_1Al_2, and the third through $M_1Am_2Al_2$.

TABLE I. RÉSUMÉ OF ATTEMPTS TO ISOLATE VIRUSES FROM PATIENTS WITH INFLUENZA, DECEMBER, 1945, TO JANUARY, 1946

NO.*	PATIENT	SERUM TITERS		DAY	DATE	VIRUS ISOLATION			
		LEE	PR8			ROUTE	RESULTS	ROUTE	RESULTS
1	M. B.	2/107	3/3	1	12/13	Am_1	4[a]/6	M_1Am_1	1[c]/1
						E_1	1/4	M_1E_1	0/1
						E_4	1[b]/1	M_1Al_1	0/2
						Al_1	0/3	M_2Am_1	1/1
						Al_3	0/1	M_2E_1	0/1
						Am_1Al_1	0/2	M_2Al_1	0/1
						Am_3Al_1	1/1	M_2Al_2	1/1
								M_3Al_1	0/1
2	D. C.	--	--	1	12/13	Am_1	1[d]/2	Am_6Al_1	1[e]/1
						Al_1	0/5	M_3Am_1	1[f]/1
						Al_5	0/1		
3	J. M.	10/44	11/11	3	12/13	Am_1	1[g]/1	Am_3Al	1[h]/1
						Al_5	0/1		
4	R. T.	4/256	14/16	3	12/13	Am_1	1[i]/2	Al_2	0/1
						E_1	0/1	Am_6Al_1	1[j]/1
5	W. D.	14/512	9/6	3	12/13	Am_1	0/4	Al_5	0/1
						Am_5	0/1	Am_1Al_1	0/1
						E_1	0/2		
6	M. F.	1/256	14/16	1	12/14	Am_1	1/2	Am_1Al_1	1[d]/2
						E_1	2[k]/2	E_1Al_1	1[l]/1
						Al_1	0/3	M_1Al_1	1[m]/1
						Al_2	0/1	M_1Al_1	1[n]/1
7	W. C.	3/44	2/2	2	12/14	Am_1	1/3[o]	Am_1Al_1	1[p]/1
						Al_1	0/1	E_1Al_2	1/1
						E_1	0/1	M_1Al_2	1[q]/1
8	M. M.	9/145	7/8	1	12/17	Am_1	1[r]/4	E_1Al_1	0/4
						E_1	0/5	M_1Am_1	1[s]/1
						E_2	0/1	M_4Am_1	1/1
						Am_1Al_1	0/2	M_1Al_2	0/2
						Am_2Al_1	1[s]/1	M_2Al_1	0/1
9	E. O.	6/28	8/7	1	12/21	Am_1	1[t]/2	Am_1Al_1	0/1
						Al_5	0/1	Am_3Al_1	1[s]/1
						E_1	0/1		
10	J. C.	4/13	10/9	1	12/25	Am_1	0/2	M_1E_1	0/1
						E_1	0/2	M_4Am_1	0/1
						M_1Am_1	0/1	M_1E_1	0/1
						M_1Al_1	0/1		
11	D. S.	6/28	8/7	2	1/4	Am_5	0/1	Al_5	0/1[s]
12	F. M.	44/44	20/60	2	1/19	Am_1	0/2	Al_2	0/1
						Am_5	0/1	E_1Al_2	0/2
						E_3	0/1		
13	L. M.	168/104	22/112	2	1/28	Am_1	0/3	E_3	0/1
						Am_5	0/1	Al_1	0/2
						E_1	0/2	Al_5	0/1
14	G. R.	10/11	2/48	2	1/31	Am_1	0/3	Al_2	0/1
						Am_5	0/2	Al_4	0/1
						E_1	0/2	Am_1Al_2	0/3
						E_4	0/1	E_1Al_1	0/1

Serum titers: average titers of complement fixation in patient's serum; acute/convalescent. Day, day of illness. Date, day when throat washing was obtained. Route: Am, amniotic; E, embryonic; Al, allantoic; M, suspension of lung from mouse inoculated intranasally; subscripts represent number of passages by that route. Results, number positive (good agglutination of hen's cells)/number attempted.

[a] One was positive in Al and negative in Am fluid. [b] Positive Al fluid, maintained through E_1Al_5. [c] Lost on subsequent E passages. [d] Positive Am and Al fluid. [e] Passage through Am_6Al_7. [f] Positive Al fluid, passed through M_3Am_4, lost in two subsequent Al passages. [g] Passage through Am_6; Al fluid from Am_1 negative; all others positive. [h] Maintained through Am_3Al_4. [i] Positive through Am_6; Al fluid positive after Am_2. [j] Maintained through Am_1Al_5. [k] Al fluid positive both times. [l] Passage through E_1Al_{21}. [m] Passage through M_1Al_{21} with wide variations in titer. [n] Nasal secretions inoculated; passage through M_1Al_4. [o] No fluid in two attempts. [p] Passage through Am_1Al_{34}. [q] Passage through M_1Al_{25}. [r] Passage through Am; Al fluid positive after Am_3. [s] Negative after Al passage; positive on further Am passage. [t] Positive Al fluid; maintained on Am passage. [u] Positive Am and Al fluids; Am_2 through Am; [v] Passage through Am_3Al_4. [s] Positive then lost in passage.

*Numbers 1 to 5 are from Needham, the others are from Boston City Hospital.

less marked or were not seen grossly after further mouse passages. On the other hand, one or more passages through mice seemed to result in a sufficient increase in concentration of virus to permit a better yield and more rapid establishment of the virus after subsequent amniotic passage in eggs. Preliminary mouse passage also permitted the establishment of the virus directly in the allantoic sac of the chick embryo on several occasions without preliminary amniotic passage.

A few details of interest and some of the difficulties encountered in the course of these isolations may be mentioned briefly. There seemed to be considerable fluctuations in the amount, or possibly the virulence, of the viruses during the course of the amniotic passages and before they were stabilized by subsequent allantoic passages. It was difficult in the early passages, for example, to judge from the hemagglutination titers the dilution of amniotic or allantoic fluid to use for subsequent passage either by the amniotic or allantoic routes. It was necessary, therefore, to use two, three, and sometimes four different dilutions of fluid for injections by both the amniotic and allantoic routes in order to assure a good virus yield and to avoid the loss of the virus in passage. Furthermore, the detection of virus in allantoic fluid, even in moderate or high titer, did not always assure subsequent successful allantoic passage even when several dilutions of virus were used. Eventually, however, it was possible to passage some of the viruses consistently by the allantoic route, although not all of the strains were carried through to that stage.

Storage of the throat washings or of passage materials in the carbon dioxide icebox did not seem to affect the viruses appreciably, at least as judged by the facility with which isolations were subsequently made from these materials. Viruses were isolated from original washings and first egg passage materials, by two individuals working independently, as long as fifteen to eighteen months after they were originally stored.

One point of particular interest was the progressive decline in titer of virus in the amniotic fluid with a concomitant increase in titer of the virus in the allantoic fluid during the course of incubation of the amniotic passages. Three striking examples of this phenomenon are shown in Table II.

TABLE II. VIRUS TITERS IN AMNIOTIC AND ALLANTOIC FLUIDS AT VARIOUS INTERVALS AFTER AMNIOTIC INOCULATION

PATIENT	SOURCE*	DAYS OF INCUBATION	CCA† TITER	
			AMNIOTIC FLUID	ALLANTOIC FLUID
E. O.	$Am_4(10^{-2})$	2	128	2
		3	<2	32
		4	<2	64
E. O.	$Am_4(10^{-3})$	2	128	16
		3	16	512
		4	2	32
R. T.	$Am_6(10^{-1})$	2	128	32
		3 (Alive)	16	64
		3 (Dead)	4	128

*Am_4 and Am_6, Fourth and sixth amniotic passages, respectively; nine-day-old embryos were used for these inoculations. The dilution of the inoculum from the previous amniotic passage is shown in parentheses.

†CCA, Chicken cell agglutination.

Identification and Differentiation of Strains.—

Human Convalescent Sera: In the course of the serologic studies of the cases from the school outbreak in Needham,[4] some evidence was obtained which suggested that there were antigenic differences among the strains of influenza virus isolated in this epidemic, although the strains were readily identified as influenza B. Most of the patients from Needham who had clinically typical influenza showed a characteristic rise in antibody titer in the convalescent sera with the Lee strain of influenza B as well as with three recently isolated strains, but not with the PR8 strain of influenza A virus.

There were three patients in that group, however, who failed to show a rise in antibodies by the agglutinin inhibition test with the Lee strain and with one of the recently isolated strains, but did show a rise with the other two epidemic strains that were used. In two of these three patients similar results were obtained with the complement fixation test, but in the third patient the complement fixation test showed similar rises in titer with all of these four strains of influenza B virus. The serologic findings in these three children and in the three patients from whom the viruses were isolated are shown in Table III.

TABLE III. ANTIBODY TITERS OF SERA FROM SELECTED PATIENTS TESTED WITH STOCK STRAINS AND RECENTLY ISOLATED STRAINS

PATIENT	PR8		LEE		BON		WC		MF		MB	
	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT
Agglutination Inhibition Tests												
W. C.	21	24	14	56	--	--	**3**	**24**	--	--	--	--
M. F.	2	3	11	115	2	16	2	40	**5**	**64**	2	64
M. B.	28	40	9	172	6	32	4	96	3	16	**6**	**128**
J. H.	16	20	3	4	3	16	2	16	2	16	2	2
R. V.	66	72	3	3	--	--	2	6	2	16	3	3
J. M.	48	48	6	6	--	--	2	4	6	16	4	4
Complement Fixation Tests												
W. C.	2	2	3	44	--	--	--	--	--	--	--	--
M. F.	2	3	4	272	2	48	3	180	**6**	**192**	3	80
M. B.	3	3	2	107	--	--	2	64	2	56	**2**	**113**
J. H.	10	8	6	8	2	24	2	12	3	10	5	7
R. V.	8	7	2	2	--	--	2	14	2	6	2	3
J. M.	11	11	10	44	--	--	10	32	10	40	12	48

Patients M. B., J. H., R. V., and J. M. were 12, 14, 11, and 17 years old, respectively, and were from Needham; W. C. and M. F. were adult workers at the Boston City Hospital.
Titers with homologous virus are shown in bold type.

On the basis of the meager evidence obtained from the serologic response in the three Needham patients, the strain MB that was isolated from another case in Needham appeared to be antigenically closely related to the Lee strain. On the other hand, strains MF and WC, which were isolated from individuals in Boston who had not been exposed to patients from Needham, differed from these two strains and were closely related to the Australian BON strain[12] of

influenza B. It is interesting to note that these differences in antigenic response to the different strains of virus were obtained in school children ranging in age from 11 to 17 years. No such specific differences in antibody response were noted in the convalescent sera of the adult patients from the Boston City Hospital.[5]

The fact that only the convalescent sera from some of the school children showed these specific differences may be due to their having had fewer previous exposures to influenza antigens, particularly to those of type B strains. This may have permitted their antibody response to express greater specificity when compared with the antibody reaction of older patients.

Immune Rabbit Sera: The titers of agglutinin-inhibiting antibodies in the rabbit sera prepared against these various strains are shown in Table IV. The results of these tests corroborate the findings in the human convalescent sera. They show that the MB strain is indistinguishable from the Lee strain of influenza B, while strains WC and MF are identical with each other and are easily differentiated by this method from the first two viruses. The WC and MF strains, furthermore, appear to be closely related to the BON strain. All of these strains were easily differentiated from the PR8 strain of influenza A virus.

The titers of antibodies for the homologous virus in the rabbit sera obtained at the time of the final bleeding are also expressed in Table IV as fold rises. This was done because the rabbits used for immunization with the MF strain had a high titer (1:128) of hemagglutinin-inhibiting activity before the immunization. The subsequent fourfold increase in antibody titer in these rabbits was less than in those which were immunized with the other strains and in which the control titers were lower. The anti-MF serum was less active than the other rabbit antisera, and the difference was even more marked in the results obtained in the virus neutralization tests with these sera.

TABLE IV. CHICKEN CELL HEMAGGLUTINATION INHIBITION TITERS OF ANTISERA PREPARED IN RABBITS AGAINST SELECTED STRAINS OF INFLUENZA VIRUS

VIRUS	TITER OF HOMOLOGOUS RABBIT SERA AT TIME OF FINAL BLEEDING			TITERS OF IMMUNE RABBIT SERA TESTED SIMULTANEOUSLY AT A LATER DATE					
	CONTROL	IMMUNE	FOLD RISE	PR8	LEE	BON	WC	MF	MB
PR8	4	**2048**	512	**512**	8	8	8	16	8
Lee	4	**1024**	256	8	**64**	8	16	16	64
BON	32	**256**	8	8	32	**64**	32	32	32
WC	16	**256**	16	4	16	16	**128**	64	16
MF	128	**512**	4	4	16	32	64	**64**	16
MB	16	**256**	16	16	128	8	16	16	**128**

Titers with homologous virus are in bold type.

The results of the virus neutralization tests performed on embryonated eggs are shown in Table V. These tests appear to be more sensitive and more specific and, therefore, give a more accurate measure of the antigenic differences. In these tests, the control or normal rabbit sera showed no neutralizing activity with any of the viruses. The results otherwise confirm the close antigenic relationships of strains MB and Lee and their differences from strains WC, MF, and BON which are closely related to each other. There are also minor but

suggestive antigenic differences between WC on the one hand and MF and BON on the other. These latter differences, however, may be due in part to the fact that the neutralizing titer of the antiserum prepared with the WC strain was appreciably higher than that of the MF and BON antisera.

TABLE V. RESULTS OF VIRUS NEUTRALIZATION TESTS IN CHICK EMBRYOS WITH IMMUNE RABBIT SERA AND SELECTED STRAINS OF INFLUENZA VIRUS

VIRUS	IMMUNE RABBIT SERUM PREPARED AGAINST:					
	PR8	LEE	BON	WC	MF	MB
PR8	>128*	<4	--	< 4	< 4	< 4
Lee	<4	>128	--	< 4	< 4	32
BON	--	8	16	16	12	< 4
WC	--	12	--	64	16	< 4
MF	<4	10	--	16	15	< 4
MB	<4	64	--	< 4	< 4	70

*Titer expressed as the reciprocal of the dilution of serum which inhibits growth in 50 per cent of embryos inoculated with 300 ID_{50} (50 per cent infecting doses).

Hemagglutination of Horse Cells: The ability of all of these strains of virus to agglutinate horse red blood cells was tested in order to detect any possible difference in activity similar to that found for some strains by Burnet.[13] There were some suggestive differences but these were not marked nor were they observed consistently in repeated tests.

Attempts to Demonstrate a Receptor Gradient: Experiments were also performed to determine whether any possible differences in these viruses could be determined by the receptor gradient analysis as described by Burnet and co-workers.[14] Each of the viruses was used to agglutinate chicken red blood cells. The viruses were then eluted from the red cells. The cells from which each of the viruses was eluted were then used for agglutination with all the viruses. No appreciable differences were noted in the effect on the red cells of any of the strains of influenza B virus that were tested in this manner.

SUMMARY AND CONCLUSIONS

Details of the isolation and identification of influenza viruses from throat washings obtained from patients with clinical influenza in and around Boston during the epidemic of December, 1945, have been presented.

Strains of influenza B virus were isolated only during the height of the epidemic from patients whose convalescent sera showed a rise in antibodies for influenza B and not for influenza A.

The amniotic route of inoculation of chick embryos was the most successful for the primary isolation of these viruses. Preliminary mouse passage was sometimes helpful in establishing the virus more rapidly in the embryonated eggs.

Evidence was presented which indicated that at least two antigenically distinct strains of influenza B were active in this epidemic.

REFERENCES

1. Finland, M., Barnes, M. W., and Samper, B. A.: Influenza Virus Isolations and Serologic Studies Made in Boston During the Winter of 1943-1944, J. Clin. Investigation **24**: 192-208, 1945.

2. Thigpen, M., and Crowley, J.: Isolation of Influenza A by Intra-allantoic Inoculation of Untreated Throat Washings, Science **98**: 516, 1943.
3. Rickard, E. R., Thigpen, M. P., and Crowley, J. H.: The Isolation of Influenza A Virus by the Intra-allantoic Inoculation of Chick Embryos With Untreated Throat Washings, J. Immunol. **49**: 263-271, 1944.
4. Finland, M., Barnes, M. W., Meads, M., Ory, E. M., and Rubenstein, A. D.: Influenza B in Needham, Massachusetts, December, 1945. Serologic Studies in Cases and in Family Contacts, J. Lab. & Clin. Med. **32**: 367-378, 1947.
5. Finland, M., Barnes, M. W., Meads, M., and Ory, E. M.: Serologic Studies of Influenza Made in Boston During the Winter of 1945-1946, J. Lab. & Clin. Med. **33**: 15, 1948.
6. Finland, M., Ory, E. M., Meads, M., and Barnes, M. W.: Influenza and Pneumonia. Serologic Studies During and After an Outbreak of Influenza B, J. Lab. & Clin. Med. **33**: 32, 1948.
7. Burnet, F. M.: Influenza Virus Infection of the Chick Embryo Lung, Brit. J. Exper. Path. **21**: 147-153, 1940.
8. Hirst, G. K.: The Quantitative Determination of Influenza Virus and Antibodies by Means of Red Cell Agglutination, J. Exper. Med. **75**: 49-64, 1942.
9. Hirst, G. K.: Direct Isolation of Human Influenza Virus in Chick Embryos, J. Immunol. **45**: 293-302, 1942.
10. Francis, T., Jr., and Salk, J. E.: A Simplified Procedure for the Concentration and Purification of Influenza Virus, Science **96**: 499-500, 1942.
11. Hirst, G. K.: In Vivo Titrations of Influenza Virus and of Neutralizing Antibodies in Chick Embryos, J. Immunol. **45**: 285-292, 1942.
12. Beveridge, W. I. B., Burnet, F. M., and Williams, S. E.: The Isolation of Influenza A and B Viruses by Chick-Embryo Inoculation, Australian J. Exper. Biol. & M. Sc. **22**: 1-7, 1944.
13. Burnet, F. M.: Red Cell Agglutination by Viruses. The Hirst Phenomenon, Australian J. Sc. **5**: 5, 1942.
14. Burnet, F. M., McCrea, J. F., and Stone, J. D.: Modification of Human Red Cells by Virus Action. I. The Receptor Gradient for Virus Action in Human Red Cells, Brit. J. Exper. Path. **27**: 228-236, 1946.

HISTAMINE ANTAGONISTS

X. A New Antihistaminic Drug, 2-[α-(2-Dimethylaminoethoxy)-α-Methylbenzyl]-Pyridine Succinate (Decapryn Succinate); Experimental and Clinical Results

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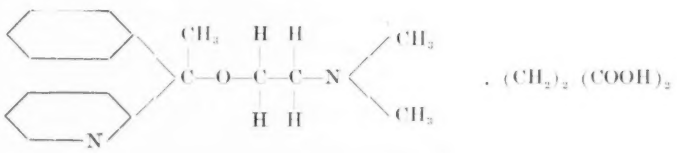
RAPID developments in the field of antihistaminic drugs have led to the synthesis of another new compound which, according to our experimental and clinical experiences, is an effective antihistaminic and antiallergic agent. This substance is 2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]-pyridine succinate, which the manufacturer calls Decapryn succinate.*



Laboratory studies by Brown and Werner[1] have demonstrated that decapryn succinate is an antihistaminic agent with comparatively high potency and low acute and chronic toxicity. Minimal doses necessary to antagonize 4 to 7 LD_{100} of histamine were reported to be less than 0.5 mg. per kilogram intravenously and subcutaneously and 10 mg. per kilogram orally; lethal doses by these routes were sixty-four or more times the effective doses. Protective action in these tests was of long duration, as evidenced by the fact that seven lethal doses of histamine were antagonized ten hours after the oral administration of 80 mg. per kilogram of the antihistaminic agent.

Decapryn succinate effectively antagonized the depressor response in cats and the pressor response in rabbits resulting from histamine. It was found to have practically no topical anesthetic action on rabbit cornea, but alkalinization of solutions liberated the base and resulted in strong local anesthetic action. The succinate also produced strong anesthetic action of long duration when injected with epinephrine. It prevented anaphylactic death in guinea pigs passively sensitized to horse serum; it antagonized the massive edema-producing effects of intraperitoneally administered egg white in rats; and both oral and topical applications antagonized in some measure the contact dermatitis induced by chemical antigens.

These data indicated to us that decapryn had properties warranting further experimental and clinical study.

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EXPERIMENTAL INVESTIGATIONS

Decapryn succinate produces very little surface anesthesia of mucous membranes. Unlike most of the other antihistaminic drugs, a few crystals on the tongue did not produce noticeable numbness. One or two per cent solutions in the rabbit's eye did not abolish the sensitivity of the cornea. However, the intradermal injection of a solution into the guinea pig indicated that it is a local anesthetic about equal in potency to novocaine.

Intravenous Histamine.—The 1 M.L.D. 100 of histamine (0.4 mg. of histamine base per kilogram) given intravenously to guinea pigs resulted in a survival of ten out of ten animals when an intraperitoneal injection of 1 mg. of decapryn succinate per kilogram was administered twenty minutes previously. When 0.1 mg. per kilogram of the drug was injected, two of five animals died after 1 M.L.D. 100 of histamine.

Aerosols in the Prevention of Histamine Bronchospasm.—We have found that aerosols of the antihistamine drugs were effective in the prevention of dyspnea from aerosolized histamine to which the guinea pigs were subsequently exposed. Ten animals were selected which responded to our histamine aerosol (0.5 mg. per cubic centimeter at 5 pounds pressure) with the production of labored breathing in one-half to three minutes. These same animals were subjected on different days to the antihistaminic drug, usually in strengths of 2, 1, ½, and ¼ per cent, for a five-minute period. After fifteen minutes the animals were exposed to the histamine aerosol and the time required to produce dyspnea was noted. If no dyspnea occurred after ten minutes the animals were removed. Periodically the animals were tested for changes in histamine tolerance and, if changes were noted, were replaced with other animals.

TABLE I. EFFECT OF DIFFERING CONCENTRATIONS OF AEROSOLS ON GUINEA PIGS TOLERATING HISTAMINE AEROSOLS (TEN ANIMALS WITH A NORMAL HISTAMINE TOLERANCE OF ONE-HALF TO THREE MINUTES)

DRUG (%)	NUMBER OF ANIMALS TOLERATING DOUBLE HISTAMINE EXPOSURE	NUMBER OF ANIMALS TOLERATING 10 MIN. HISTAMINE EXPOSURE
Pyribenzamine		
2	10	10
1	10	10
½	10	9
¼	6	1
Decapyrn succinate		
2	9	6
1	5	1
½	5	0
¼	7	
3015 RP		
2	8	3
1	2	0
½	3	0
¼	0	0
Antistine		
2	10	1
1	6	1
½	5	0
¼	0	0

The essential results with decapryn succinate in comparison with Pyribenzamine and two other drugs are briefly reported in Table I. It may be seen that while decapryn succinate as an aerosol is not so effective as some antihistaminic drugs, it is more effective than others.

Anaphylaxis.—Some degree of protection against anaphylaxis was evidenced by the fact that of nine animals passively sensitized with antiegg serum and challenged with 0.5 c.c. of 10 per cent egg white intracardially only one died of anaphylaxis when an intraperitoneal injection of 3 mg. of decapryn succinate per kilogram was used thirty minutes previously. With a 1 mg. per kilogram dose the protection was practically nil. The control group showed a mortality of 85 per cent.

Inhibition of Local Histamine Skin Response in Man.—Early in our experience with the antihistaminic drugs we demonstrated that when applied locally to skin scratches these substances were highly effective in inhibiting the wheal and flare of histamine from subsequent application. Since it is reasonable to suppose that the histamine skin response in man comes much closer than animal assays to simulation of the mechanism of the allergic phenomena which we attempt to ameliorate, it was hoped that quantitative studies of the inhibiting action of this histamine reaction might be a valuable means of obtaining a preliminary assay of the drug in question. At this point we shall not go into detail concerning the various techniques and the modifications which we employed nor

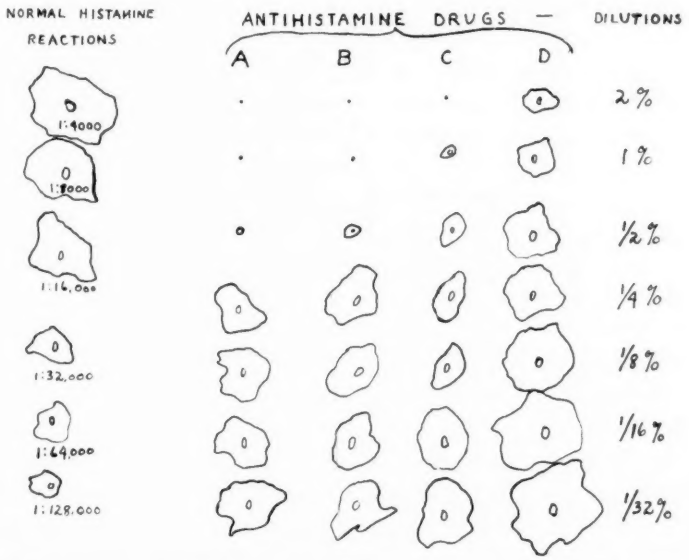

Fig. 1.—Quantitative inhibition of histamine reaction. Suitability and sensitivity of the particular subject are determined by preliminary titration of histamine dilutions applied to a series of scratches on the back (column at left). Dilutions of antihistaminic drugs—*A*, Pyribenzamine, N'pyridyl-N'benzyl-N-dimethylethylenediamine hydrochloride; *B*, Decapryn succinate, 2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]-pyridine succinate; *C*, Chlorothen, N,N-dimethyl-N'-(2-pyridyl) N'-(5-chloro-2-thenyl) ethylenediamine hydrochloride; *D*, Hetramine, N,N-dimethyl-N'benzyl-N' (α-pyrimidyl) ethylenediamine hydrochloride—are applied to several rows of scratches on the same subject at the same visit. The drugs are washed off ten minutes later and 1 drop of a selected concentration of histamine (in this case 1:4,000) is applied to each of the scratches. The comparative inhibitive effect of the drug gives a good indication of its antiallergic efficacy. The procedure is repeated on a number of subjects. Other modifications of the technique based on the same concept are being used.

discuss our general data with respect to other drugs. We shall outline here one method which can be used to obtain data and also the results obtained with decapryn succinate.

The suitability and the sensitivity of the subject are determined by performing tests with serial dilutions of histamine on scratches on the back. The dilutions commonly employed range from 1:4,000 to 128,000 in terms of histamine base. The concentration which is twice that of the lowest giving the maximum flare is usually employed in the inhibition experiments. Several series of scratches are made on the back. On each series of scratches drops of an antihistaminic substance of serial dilutions are placed, the range varying from 2 per cent to $\frac{1}{32}$ per cent.* After ten minutes the antihistaminic solutions are washed off and the selected concentration of histamine is applied to the pretreated sites. Inhibition of the histamine reaction is shown in the sites which had the more concentrated antihistaminics, while the sites which had the weak solution may react. The point at which a histamine reaction fails to be inhibited gives an idea of the antihistaminic and probably the antiallergic potency of the drug. The experiment is, of course, repeated on a number of subjects.

Fig. 1 gives an idea of a representative result and indicates that decapryn succinate is effective in the inhibition of histamine reactions.

CLINICAL FINDINGS

General Procedure.—After some of the experimental work had been done, the drug was administered cautiously to several subjects. When no particular toxic effects were noted, decapryn succinate began to be used for the symptomatic relief of allergy. The patient was given the drug in the form of capsules or tablets and instructed to use small doses at first and only when needed. In a few instances the medication was prescribed to be taken regularly two to four times daily. In most instances the patient was seen twice weekly, when his condition was evaluated by questioning and observation and judged in terms of pollen counts, weather changes, and similar factors. Whenever feasible the subject was asked to compare the action of this drug against one or more others at various times. The results were regarded as satisfactory if relief was consistently obtained, although not necessarily every time. If relief was very slight or questionable or not obtained most of the time the result was recorded as "no improvement."

Hay Fever and Nonseasonal Allergic Rhinitis.—The seasonal group comprised eighty-one patients, observed during the 1947 season, having seasonal hay fever, either due to the pollens of grasses or ragweed or to fungus spores. Subjects with hay fever due to ragweed predominated. While some of the patients to whom this drug was given were new or untreated, the majority had received previously varying amounts of desensitizing injections. Satisfactory relief was obtained in sixty-two of the persons with hay fever (76 per cent).

*More recently we have found the following factors more suitable: histamine range of 1:8,000 to 1:512,000 for control titration; concentration of histamine for inhibition either the first effective flare or one which is one-half size of maximal; and antihistamine concentrations ranging from 1:800 to 1:204,800.

The relief was, of course, only temporary. The duration of action is difficult to determine in hay fever; our impression, however, is that there was a tendency to longer action than found with other antihistaminic drugs.

In the nonseasonal vasomotor rhinitis, nineteen of thirty-four patients obtained satisfactory improvement. In both the seasonal and nonseasonal types of rhinitis the hyperesthetic symptom of sneezing was more often relieved than the nasal blocking. The intumescence of the turbinates, so common a manifestation of the latter part of the hay fever season, was resistant to this drug as well as to other antihistaminic drugs.

Other Allergic Manifestations.—No appreciable relief could be observed in any of twenty-seven patients with asthma. Most of the patients with asthma in this group were associated with hay fever. This fact cannot be overstressed. In hay fever asthma, the hay fever may be effectively relieved, whereas the asthma is not. Since desensitization treatment is highly effective in the prevention of such asthma, it is obvious that antihistaminic therapy should not be depended upon in such cases. Two patients of seven had relief of allergic cough.

The itching and some of the swelling of urticaria and angioneurotic edema were relieved in six patients of nine treated. Six patients with chronic atopic dermatitis received decapryn succinate; four had appreciable relief of itching. Dermographism was effectively relieved in five of six patients. Two patients with headache of possible allergic origin failed to obtain benefit.

Doses.—The doses administered varied from 12.5 to 50 milligrams. We found no one who failed to tolerate as little as 12.5 mg., and doses larger than 50 mg. usually either were not needed or were not tolerated well. It is true that some patients who failed to obtain relief from 50 mg. doses might have obtained benefit from larger amounts. It is also true that some of those who obtained results from 25 mg. doses without toxic effects may have been relieved by smaller doses. This is a phase of therapy which will have to be decided in the future. However, it is well to emphasize here that decapryn succinate has in some patients a highly soporific action and large doses should not be prescribed to an individual without previous trial of smaller doses.

Of the sixty-two patients with hay fever who obtained relief, thirty did so with 25 mg. doses and 3 with 12.5 milligrams.

Side Actions.—Of one hundred eighteen patients, side actions to the drug were observed in thirty-nine (34 per cent). Of this number, sedation or sleepiness was seen in thirty-six. In some patients this sedative effect was very pronounced. In one instance sleepiness and a feeling of numbness lasted from twenty-four to thirty-six hours. Usually, however, the sedative effect did not prevent the use of the drug. Nervousness was noted in four patients and vertigo also in four. Two patients complained of headache, one of epigastric pain, and another of an itching rash. No serious or remote toxic effects have been noted after six months' use of the drug.

SUMMARY

The properties of a new antihistaminic drug, 2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]-pyridine succinate (decapryn succinate), are described.

This drug is highly effective in the prevention of histamine shock in guinea pigs and in the prevention of anaphylaxis.

It is active as an aerosol in the prevention of bronchospasm in guinea pigs from aerosolized histamine.

Its quantitative activity locally in the inhibition of the histamine reaction in man indicates that this drug is a potent antihistaminic.

Clinically this drug is highly effective in some of the allergic manifestations, particularly hay fever, chronic allergic rhinitis, and some of the itching dermatoses. Its effectiveness in asthma is negligible.

The doses employed have been mainly 25 to 50 milligrams.

Side actions occurred in 34 per cent of the subjects, sleepiness being the outstanding effect. Its soporific action is greater than that of pyribenzamine, but probably less than Benadryl in therapeutic doses. Because of marked individual susceptibilities the initial dose of the drug should not exceed 25 milligrams.

REFERENCE

1. Brown, B. B., and Werner, H. W.: The Pharmacologic Properties of 2-[α-(2-Dimethylaminoethoxy)-α-Methylbenzyl]-Pyridine Succinate, A New Antihistaminic Agent, J. Lab. & Clin. Med. 33: 325, 1948.

THE PHARMACOLOGIC PROPERTIES OF 2-[α-(2-DIMETHYLAMINOETHOXY)-α-METHYLBENZYL]-PYRIDINE SUCCINATE, A NEW ANTIHISTAMINIC AGENT

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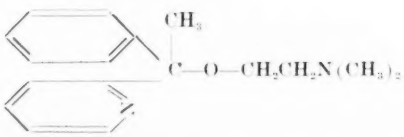
WITH THE TECHNICAL ASSISTANCE OF EMMETT L. PETERS

THE original discovery by Bovet and Staub[1, 2] that certain Fourneau compounds possess activity antagonistic to histamine has led to an intensive search for other compounds with greater effectiveness and safety, and investigations in this country by Loew[3] and Mayer[4] and their co-workers have resulted in the trial and introduction of two compounds, diphenhydramine hydrochloride (Benadryl) and triplennamine hydrochloride (Pyribenzamine), into clinical medicine.

The literature on these and other antihistaminic compounds is growing rapidly, and this paper presents the results of studies on another new compound which is of interest because it possesses relative high activity and low toxicity. This new substance, under the trade name Decapryn Succinate, is being evaluated clinically by several groups. Chemically it is 2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]-pyridine succinate, and the base has the following structure.



The succinate salt was used in all but one of the pharmacologic studies, and for convenience it is referred to as 2-PS. 2-PS occurs as somewhat bitter white crystals which are soluble in three parts of water.

Acute Toxicity.—Acute LD_{50} values of 2-PS for various routes of administration and different species are summarized in Table I.

Lethal or near lethal doses of 2-PS produced convulsions in all species. These were followed in rabbits, which received lethal amounts, by skeletal muscle rigidity. Deaths in all species usually occurred within two hours, but in a few instances they were delayed up to twenty-four hours.

The possibility of a toxicity difference due to sex was investigated in rats in view of the reported difference in triplennamine toxicity for this species following subcutaneous administraton.[6] The LD_{50} values of 2-PS obtained for male and for female rats (Table I) were similar. Male and female mice also

From the Pharmacology Department, Research Laboratories, The Wm. S. Merrell Company.

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TABLE I. LD_{50} VALUES AND STANDARD ERRORS FOR 2-PS ESTIMATED BY THE METHOD OF MILLER AND TAINTER[5]

ROUTE OF ADMINISTRATION	MICE (MG./KG.)	RABBITS (MG./KG.)	MALE RATS (MG./KG.)	FEMALE RATS (MG./KG.)
Intravenous	62 ± 4	49 ± 1.4	--	--
Subcutaneous	460 ± 52	--	440 ± 34	445 ± 38
Oral	470 ± 32	250 ± 42	--	--

responded similarly to toxic doses of 2-PS administered either orally or subcutaneously. Oral doses of 500 mg. per kilogram killed seven of ten male mice and six of ten female mice; while a subcutaneous dose of 470 mg. per kilogram killed seven of ten male mice and nine of ten female mice.

Isolated Guinea Pig Intestine.—A preliminary estimation of antihistaminic activity was obtained using the isolated intestinal strip method. Two- to three-centimeter strips of guinea pig intestine were suspended in 100 c.c. baths of aerated Tyrode's solution maintained at 38 ± 0.5° C. Histamine diphosphate was added to the bath to give a concentration of 0.1 μg. per cubic centimeter. The contraction was recorded and the strip was washed several times with Tyrode's solution. Each strip was tested repeatedly in this manner until three successive identical contractions were obtained. The minimal concentration of 2-PS required to inhibit the action of the standard dose of histamine was then determined.

It was found that a dilution of 0.05 μg. of 2-PS per cubic centimeter completely inhibited the response of the intestinal strip to the standard histamine dose, and dilutions as low as 0.01 μg per cubic centimeter caused partial inhibition.

Protection Against Histamine Toxicity in Guinea Pigs.—The protective action of 2-PS against the lethal effects of histamine was measured in the guinea pig by a method similar to that of Staub.[2]

The lethal dose (LD_{100}) of histamine diphosphate for guinea pigs employed at different periods in these studies, determined by injecting histamine intravenously in increasing doses at ten-minute intervals until death occurred, was found to be 0.6 mg. per kilogram. The amount of histamine antagonism was measured by determining the number of lethal doses of histamine tolerated by guinea pigs pretreated with 2-PS. The administration of 2-PS was followed by repeated injections of histamine, the dose being increased by 20 per cent for each injection until the lethal dose was reached. The histamine injections were begun ten minutes after intravenous administration and thirty-five to forty-five minutes after subcutaneous or oral administration of 2-PS in all evaluations of potency. The antihistaminic potency of 2-PS obtained by this method is tabulated in Table II.

Each value in Tables II and III represents an average of ten to sixteen determinations. The maximal activity of 2-PS was exhibited after intravenous administration. On the average, doses of 8 to 32 mg. per kilogram protected against 133 to 200 fatal doses of histamine, and in some individual animals protection was afforded against 320 lethal doses of histamine. A high degree

TABLE II. PROTECTION AGAINST HISTAMINE TOXICITY IN THE GUINEA PIG FOLLOWING INTRAVENOUS, SUBCUTANEOUS, AND ORAL ADMINISTRATION OF 2-PS

DOSE OF 2-PS (MG./KG.)		AVERAGE NUMBER OF LETHAL DOSES OF HISTAMINE TOLERATED		
I.V. AND S.C.	ORALLY	2-PS, I.V.	2-PS, S.C.	2-PS, ORALLY
0.5		5.5	4	--
1		8	6	--
2		18	8.5	--
4		42	27	--
8	10	133	51.5	7
16	20	160	55	11
32	40	200	107	40
64	80	2-PS Fatal	110	51
128	160	--	2-PS Fatal	82
--	320	--	--	93
--	640	--	--	143
				2-PS Fatal to 5 of 10 animals

of activity was observed also following subcutaneous and oral administration. Doses of 32 mg. per kilogram subcutaneously and 320 mg. per kilogram orally protected against roughly 100 lethal doses of histamine.

TABLE III. DURATION OF PROTECTION AGAINST HISTAMINE TOXICITY IN THE GUINEA PIG FOLLOWING INTRAVENOUS, SUBCUTANEOUS, AND ORAL ADMINISTRATION OF 2-PS

TIME INTERVAL AFTER 2-PS ADMINISTRATION (HR.)	AVERAGE NUMBER OF LETHAL DOSES OF HISTAMINE TOLERATED			
	2-PS, I.V. (32 MG./KG.)	2-PS, S.C. (32 MG./KG.)	2-PS, ORALLY (80 MG./KG.)	2-PS, ORALLY (160 MG./KG.)
1	90	--	--	--
2	43	70	60	135
4	2	10	38	60
6	--	9	38	46
8	--	--	14	--
10	--	--	7	--

Small doses of 2-PS by all routes antagonized the lethal effects of histamine, and the high potency of 2-PS over a wide range of dosage gives it a favorable ratio of activity to toxicity. Table II indicates that the minimal effective dose is less than 0.5 mg. per kilogram intravenously or subcutaneously and 10 mg. per kilogram orally; while lethal doses are approximately 64 mg. per kilogram intravenously, 128 mg. per kilogram subcutaneously, and 640 mg. per kilogram orally.

Table III shows that the action of 2-PS was relatively long following all routes of administration. An intravenous dose of 32 mg. per kilogram antagonized 43 lethal doses of histamine two hours after injection and 2 lethal doses four hours after injection. The duration of antagonistic action was especially long following oral administration. Effects of 38 lethal doses of histamine were antagonized six hours after 80 mg. of 2-PS per kilogram and activity was still evident ten hours after administration, at which time 7 lethal doses were antagonized.

Antagonism of Histamine Depressor Action in Cats.—The effects of 2-PS on the depressor action of histamine were investigated in sixteen cats anesthetized with Amytal Sodium.

Generally, before the administration of 2-PS, intravenous doses of histamine diphosphate smaller than 0.125 μg. per kilogram produced no significant effect on blood pressure. Doses between 0.125 and 2 to 8 μg per kilogram, in individual experiments, produced graded depressor responses. Larger doses of histamine did not increase the intensity of the depressor response.

After the intravenous injection of the antihistamine, 2 mg. per kilogram, effects of 1 to 4 μg of histamine diphosphate per kilogram were completely blocked; however, with increasing doses of histamine, graded depressor responses were again obtained. This is illustrated by results of a typical experiment summarized in Table IV.

TABLE IV. DEPRESSOR RESPONSE OF AN ANESTHETIZED CAT TO HISTAMINE DIPHOSPHATE BEFORE AND AFTER 2 MG. OF 2-PS PER KILOGRAM INTRAVENOUSLY

HISTAMINE DIPHOSPHATE (I.V., μG./KG.)	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Blood pressure drop in mm. Hg												
Before 2-PS	12	22	26	34	38	54	50	54	70	--	--	--
After 2-PS	0	0	0	0	0	22	21	30	40	42	44	44

Table V shows the increase in dosage of histamine required, after varying amounts of 2-PS, to produce responses equivalent to those originally produced by 0.5 μg per kilogram in individual animals. The data indicate that maximal antagonistic effects were obtained with 2 mg. of 2-PS per kilogram. According to the evaluation of Wells and co-workers,[7] an increase in dose from 0.5 μg before to 8 μg after an antihistamine indicates that 7.5 μg of histamine have been antagonized. This represents an antagonism of approximately 95 per cent of the administered histamine. A nearly similar degree of antagonism occurred in one of two animals receiving 0.5 mg. per kilogram, in one of two receiving 1.0 mg. per kilogram, and in all animals receiving higher doses of 2-PS.

TABLE V. HISTAMINE DIPHOSPHATE PRODUCING DEPRESSOR RESPONSES AFTER 2-PS EQUAL TO THOSE PRODUCED BY 0.5 μG OF HISTAMINE DIPHOSPHATE PER KILOGRAM BEFORE 2-PS ADMINISTRATION

DOSE OF 2-PS (I.V., MG./KG.)	0.05 AND 0.2	0.5	1.0	2.0	4.0 AND 8.0
Doses of histamine diphosphate, I.V., in μg/kg. equivalent to 0.5 μg/kg. before 2-PS	0.5	3	4	8	6
	0.5	8	8	8	8
	1.0			11	
	2.0			13	
				14	
				14	

Incidental to the antagonism study in cats, it was noted that the blood pressure response changed from depressor to pressor following 2 to 4 mg. or more of histamine diphosphate per kilogram when animals survived these doses.

Antagonism of Histamine Pressor Response in Rabbits.—The antagonistic action of 2-PS on the pressor action of histamine diphosphate was evaluated in twelve rabbits anesthetized with sodium phenobarbital. It was found that in this species similar doses, ranging between 0.25 and 0.4 mg. per kilogram, generally produced pressor responses of the same order of magnitude.

The intravenous administration of 1 to 2 mg. per kilogram of the hydrobromide of 2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]-pyridine completely blocked the pressor effects of small doses of histamine, and the amount of histamine required to produce pressor responses obtained before administration of the antihistamine was generally increased about thirtyfold.

Results of these experiments in rabbits and the experiments in cats indicate that 2-PS antagonizes histamine-induced arteriolar constriction in rabbits as well as vascular dilatation in cats.

Antagonism of Histamine Skin Wheals.—The histamine whealing response was produced in rabbits according to the procedure of Rigdon.[8] Dilutions of 1:1,000, 1:10,000, and 1:100,000 of histamine diphosphate were injected intracutaneously in 0.2 c.c. volumes followed in five minutes by the intravenous injection of 10 c.c. of 0.2 per cent trypan blue. Wheals developed within fifteen minutes and were characterized by an edematous area surrounded by an extravasation of blue dye indicating increased capillary permeability.

The effect of 2-PS on the whealing response was determined at various intervals following oral administration. A total of fifty-six rabbits was used in seven experiments. Untreated rabbits served as controls, and each animal received 0.2 c.c. of saline intracutaneously for comparison with the histamine injections.

The oral administration of 75 mg. of 2-PS per kilogram entirely prevented the extravasation of dye at sites injected with histamine throughout the eight-hour observation period. 2-PS also inhibited blanching for at least six hours. A smaller dose of 40 mg. per kilogram given orally completely inhibited blanching and dye extravasation for one hour and prevented the blue extravasation for from four to six hours.

Local Anesthesia.—The topical anesthetic activity of 2-PS was evaluated in the usual manner on the cornea of the rabbit, and it was found that concentrations up to 4 per cent produced no anesthesia. The absence of anesthesia apparently resulted from a failure of the salt to penetrate, since a 1 per cent solution adjusted to a pH of 9.43 produced deep anesthesia for sixteen minutes.

The local anesthetic property of 2-PS was further demonstrated in rabbits, employing a method described by Tatum.[9] It was found in control experiments that the subcutaneous infiltration of 0.33 c.c. of 2 per cent procaine near the end of the external canthus and under the lateral portions of the upper and lower lids blocked the lid reflex for an average of sixty-two minutes. Identical amounts of 2-PS produced a block for six and one-half minutes. Alkalinization of 2 per cent solutions of 2-PS to a pH of approximately 9 liberated the base and increased the duration of anesthesia to twenty-three minutes. The inclusion of 1:25,000 epinephrine hydrochloride in this solution further increased the duration of action to over five hours.

Antispasmodic Activity.—The antispasmodic action of 2-PS in vitro was measured on the isolated rabbit intestine according to the method of Magnus. The data in Table VI indicate that 2-PS has some acetylcholine antagonizing action, but this action is weak compared with that of atropine. The activity of 2-PS against a spasm induced by barium ions approaches that of papaverine.

TABLE VI. ANTISPASMODIC ACTIVITY OF 2-PS, ATROPINE, AND PAPAVERINE ON ISOLATED RABBIT JEJUNUM

COMPOUND	MINIMAL EFFECTIVE CONCENTRATION		
	NORMAL INTESTINE	VS. ACETYLCHOLINE (1:1,000,000) SPASM	VS. $BaCl_2$ (1:10,000) SPASM
2-PS	1:50,000	1:50,000	1:100,000
Atropine	1:10,000,000	1:80,000,000	1:200,000
Papaverine	1:150,000	1:100,000	1:150,000

Antiacetylcholine action was further studied in cats anesthetized with Amytal Sodium. Intestinal activity was recorded by a tambour connected to a balloon inserted into the jejunum, with a water manometer in the system to regulate intestinal pressure. Blood pressure was recorded in the usual manner from the carotid artery. The intestinal responses were standardized by repeated intravenous injections of 0.01 mg. of acetylcholine per kilogram, and no preparation was used unless identical consecutive responses were obtained. 2-PS was then injected intravenously and was followed by the standard acetylcholine injections at two- to four-minute intervals. 2-PS in a dose of 4 mg. per kilogram antagonized acetylcholine action on the intestine by approximately 50 per cent. Twice this dose produced a slightly greater inhibition. The effect of 2-PS on the blood pressure response to acetylcholine was not evaluated since in these experiments the acetylcholine doses employed were far above minimal doses required for maximal responses in the blood pressure.

Blood Pressure and Respiration.—Intravenous injections of 2-PS in cats anesthetized with Amytal Sodium, given at a rate of 2 mg. per minute, produced a slight temporary pressor effect with doses of 2 mg. per kilogram and either a slight pressor or depressor effect with doses of 4 and 8 mg. per kilogram. A rapid rate of injection of 20 mg. per minute produced a transient fall in blood pressure with doses of 8 mg. per kilogram and a slight pressor or depressor response with 2 and 4 mg. per kilogram.

Respiration generally was increased temporarily in both rate and depth with doses of 4 and 8 mg. per kilogram regardless of the rate of injection. Smaller doses had no appreciable effect.

Irritation.—2-PS produced no signs of irritation or tissue damage following either one-minute instillation in rabbits' eyes of solutions up to 4 per cent or the injection of 1 per cent solution subcutaneously in guinea pigs and intramuscularly in rats. Subcutaneous and intramuscular injections of 3½ and 4 per cent solutions in rats produced irritation and some necrosis.

SUMMARY

Experiments in laboratory animals demonstrate that 2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]-pyridine succinate (2-PS) has a comparatively low toxicity and has potent antagonistic action to the effects of histamine on various tissues.

It suppresses bronchial constriction resulting from intravenous injections of histamine in guinea pigs. Following intravenous administration, it antagonizes an average of up to 200 lethal doses of histamine and prevents death

in some animals from 320 lethal doses. A high degree of antagonistic action also follows subcutaneous and oral administrations in guinea pigs, and the protective action is of long duration.

2-PS effectively antagonizes arteriolar constriction and pressor effects of histamine in rabbits and it also antagonizes the depressor action of histamine in cats. Effective doses of histamine for these two vascular effects are increased roughly fifteen to thirty fold.

Cutaneous effects of histamine, as measured by the whealing reaction in rabbits, are antagonized. The increased capillary permeability demonstrated by localization of intravenously injected dye is prevented for eight or more hours following a single dose of 2-PS orally.

2-PS has considerable local anesthetic activity, and it is weakly antagonistic to acetylcholine.

REFERENCES

1. Bovet, D., and Staub, A. M.: Action protectrice des éthers phénoliques au cours de l'intoxication histaminique, Compt. rend. Soc. de Biol. **124**: 547, 1937.
2. Staub, A. M.: Recherches sur quelques bases synthétiques antagonistes de l'histamine, Ann. Inst. Pasteur. **63**: 400, 1939.
3. Loew, E. R., Kaiser, M. E., and Moore, U.: Synthetic Benzhydryl Alkamine Ethers Effective in Preventing Fatal Experimental Asthma in Guinea Pigs Exposed to Atomized Histamine, J. Pharmacol. & Exper. Therap. **83**: 120, 1945.
4. Mayer, R. L., Huttrer, C. P., and Scholz, C. R.: Antihistaminic and Antianaphylactic Activity of Some α-Pyridinoethylenediamines, Science **102**: 93, 1945.
5. Miller, L. C., and Tainter, M. L.: Estimation of the ED_{50} and Its Error by Means of Logarithmic-Probit Graph Paper, Proc. Soc. Exper. Biol. & Med. **57**: 261, 1944.
6. Mayer, R. L., Hays, H. W., Brousseau, D., Mathieson, D., Renniek, B., and Yonkman, F. F.: Pyribenzamine (N′-Pyridyl-N′-Benzyl-N-Dimethylethylenediamine HCl), An Antagonist of Histamine, J. Lab. & Clin. Med. **31**: 749, 1946.
7. Wells, J. A., Morris, H. C., Bull, H. B., and Dragstedt, C. A.: Observations on the Nature of the Antagonism of Histamine by β-Dimethylaminoethyl Benzhydryl Ether (Benadryl), J. Pharmacol. & Exper. Therap. **85**: 122, 1945.
8. Rigdon, R. H.: Observations on the Effect of Histamine Phosphate on Capillary Permeability and Inflammation, J. Lab. & Clin. Med. **27**: 1554, 1942.
9. Tatum, A. L.: Pharmacology Laboratory Experiments, University of Wisconsin.

THE EFFECTS OF ADRENALIN IN NORMAL AND HYPERTENSIVE PATIENTS IN RELATION TO THE MECHANISM OF SUSTAINED PRESSURE ELEVATIONS

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THE enigma of hypertension is not alone that the pressure rises but also that it fails to be restored to its former level. Many internal and external agents are capable of elevating the human blood pressure. Ordinarily, homeostatic mechanism induce a reversion to normal. The existence of sustained hypertension implies that the restorative mechanisms are either ineffective or in restraint.

One of the most important of these mechanisms is constituted by the moderator nerves, the interruption of which can produce a prolonged elevation in pressure. Investigations of the effect of carotid sinus stimulation by Weiss and Baker,[1] Moore and Allen,[2] and Thomas[3] have demonstrated that this mechanism is capable of greater depressor activity in the hypertensive person than in the normal person, indicating that sustained hypertension is not due to a reduction in the effectiveness of moderator action on peripheral structures. This capacity for vascular relaxation has been confirmed by a number of independent methods. Allen and co-workers[4] and Gregory and Levin[5] have reported that the pressure of hypertensive patients is reduced to values approaching normal by spinal anesthesia. Chasis and associates[6] induced prolonged remissions by utilizing the vasodilator action of parenteral pyrogens. More recently, the tetraethylammonium ion, like carotid sinus stimulation, has been shown by Berry and co-workers[7] to cause a pressure fall which is of greatest intensity in subjects with elevated pressure.

The concept of relatively ineffective homeostatic mechanisms is suggested by tests designed to show that hypertensive persons react excessively to pressor stimuli. Perhaps best known is the cold pressor test of Hines and Brown,[8] the use of which has been extended to single out prehypertensive individuals. However, exaggerated responses could not be demonstrated consistently in hypertensive patients by Pickering and Kissin[9] and were found absent in hypertension due to chronic renal disease by Miller and Bruger[10] and Alam and Smirk.[11] The use of the test to predict the subsequent occurrence of hypertension in pregnancy was attempted without success by Chesley and Chesley[12] and Wellen.[13] An increased incidence of hyperreaction with age was demonstrated for both normal and hypertensive subjects by Russek and Zohman,[14] while marked variability of response on repetition was reported by Goldring and Chasis in studies conducted on the same subjects over a period of years.[15, p. 17] The sum

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of these investigations indicates that a general relation between the level of blood pressure and the intensity of the cold pressor response has not been demonstrated.

The average pressor response to breath-holding was reported by Ayman and Goldshine[16] to be two to four times greater in hypertensive persons than in normal individuals. However, 20 per cent of their normotensive subjects were hyperreactors. Feldt and Wenstrand[17] compared the reactions to breath-holding and cold in the same individuals. Correlation was absent in more than 25 per cent of the group.

The interpretation of the cold pressor, breath-holding, and similar tests is complicated by the reflex nature of their action. As a consequence, variations in the pattern of response cannot be ascribed primarily to differences in the capacity of the cardiovascular apparatus to compensate for a fixed stimulus, but may be due rather to alterations in the intensity and duration of the discharge induced by the various types of reflex pressor stimuli.

The present study was designed to determine whether the pattern of response of the hypertensive person differed significantly from that of the normal individual when evoked by a pressor stimulus which by-passed the initial reflex arc. Adrenalin was chosen as the stimulus not only for its direct action on heart and vessels but also because of its rapid inactivation. It was decided to give the drug by continuous infusion at various rates, since this type of administration allows better dosage control, avoids differences due to rate of absorption and local sensory stimulation, and facilities equilibrium between the pressor actions of the drug and the depressor mechanisms of the subject.

Previous investigations of the action of adrenalin as modified by the initial pressure have been productive of varied results. The subcutaneous injection of 1 mg. was reported by Clough[18] to produce a greater incidence of excessive reaction in hypertensive subjects. However, these reactions did not seem to be related to the type, extent, or duration of the pressure elevation. On the contrary, intravenous administration of single doses by Hougardy[19] produced responses of the same magnitude in normal dogs as in those whose pressures had been significantly lowered by adrenal decapsulation. Continuous infusion of adrenalin at the rate of 2 mg. per hour was reported by Koehler and co-workers[20] to produce a rise in systolic pressure but a fall in diastolic pressure in both normal and hypertensive persons. Both the systolic rise and the postinfusion fall in pressure were said to be greater in subjects with elevated pressure.

EXPERIMENTAL METHOD

A total of eighty studies was carried out on thirty-nine male and twelve female subjects whose blood pressures ranged from 90 to 246 mm. Hg systolic and from 55 to 126 mm. diastolic. The age distribution is detailed in Table I.

Initial blood pressure and pulse values were established during the administration of saline through a three-way stopcock connected to a calibrated drip apparatus. The stopcock was turned to permit infusion of the adrenalin solution from a flask suspended 200 cm. above the individual.

Pulse and blood pressure values were determined every three to five minutes during the course of the infusion, every one minute for the first fifteen minutes after the infusion, and at lengthening intervals throughout the next twelve hours.

TABLE I. AGE DISTRIBUTION OF ADRENALIN INFUSION SUBJECTS

AGE (YR.)	NUMBER OF SUBJECTS
30-39	6
40-49	15
50-59	7
60-69	12
70-79	11

Samples of blood were drawn for determination of the hematocrit value before infusion, again when the initial dose had produced its maximum effect, and finally at the point of minimum blood pressure following cessation of the infusion.

The adrenalin solution was made up immediately before use by diluting a standard U. S. P. stock solution with isotonic saline to a final concentration of 1.0 mg. per cent. A fresh bottle of the stock solution was used for each six patients. Normotensive and hypertensive patients were treated alternately. From time to time different stock solutions were checked against one another on the same patient. No significant variations in activity were noted.

The earlier subjects in the series were given adrenalin at an initial rate of 0.31 to 0.50 μg per kilogram per minute. Later the initial rate was lowered to a value lying between 0.11 and 0.30 μg per kilogram per minute since unpleasant side effects were encountered occasionally with the higher dosage.

When the starting level had produced its maximum effect on blood pressure, the rate of administration was increased in steps of 0.20 μg per kilogram per minute until the limit of tolerance of the individual patient was reached. The infusion was maintained at this level until the end of a one and one-quarter hour period.

Tolerance and Untoward Effects.—Tolerance was inversely proportional to the initial blood pressure. In the majority of instances, maximum tolerance was determined by the occurrence of unpleasant subjective reactions, including anxiety, tremor, heart consciousness, and headache. One subject in the series complained of mild substernal pain which subsided without sequelae when the infusion was stopped. Cardiac irregularity, due to premature contractions, occurred to some degree in nearly all patients. In one instance it was sufficiently marked to dictate the cessation of the infusion.

Three persons developed cerebral symptoms, characterized in one by intense headache and momentary syncope, in another by transient unconsciousness, and in the third by a hemiplegic syndrome without loss of consciousness, from which recovery was complete in twelve hours. In two of these instances the reaction occurred during administration of the initial dose of adrenalin. These alarming episodes, which resembled the encephalopathic manifestations seen in pheochromocytoma,[21] occurred during the later phase of these studies and influenced their termination.

Analytic Methods.—The subjects were divided into four groups on the basis of the initial diastolic blood pressure level. The pressure range from 51 to 70 mm. Hg included thirteen subjects; 71 to 90 mm., seventeen subjects; 91 to 110 mm., sixteen subjects; and 111 to 130 mm., five subjects.

The mean group values of the blood pressure, pulse rate, change in pressure and pulse rate, and the systolic-diastolic pressure ratio were computed for each range of dosage tolerated by two or more of the subjects in a group. The pressure and pulse values which corresponded to the highest systolic pressure observed at each dose level were used in making computations.

Variability of response was estimated in terms of the standard deviation from the mean, corrected for small samples, while the significance of differences between groups were determined by calculation of the t value, which indicate the probability that the observed differences did not arise by chance.

The presence of linear relationship between variables was evaluated by calculation of the correlation coefficient.

RESULTS

The sequence of events in the course of an adrenalin infusion is illustrated in Fig. 1. During the first few minutes a period existed in which the incoming adrenalin solution was diluted by the saline in the tubing which connected the stopcock to the infusion needle. In this period the systolic pressure, diastolic pressure, or both ordinarily manifested a fall, followed by a sharp rise when undiluted solution reached the circulation. This rise usually elevated the systolic and occasionally the diastolic pressure above the preinfusion value.

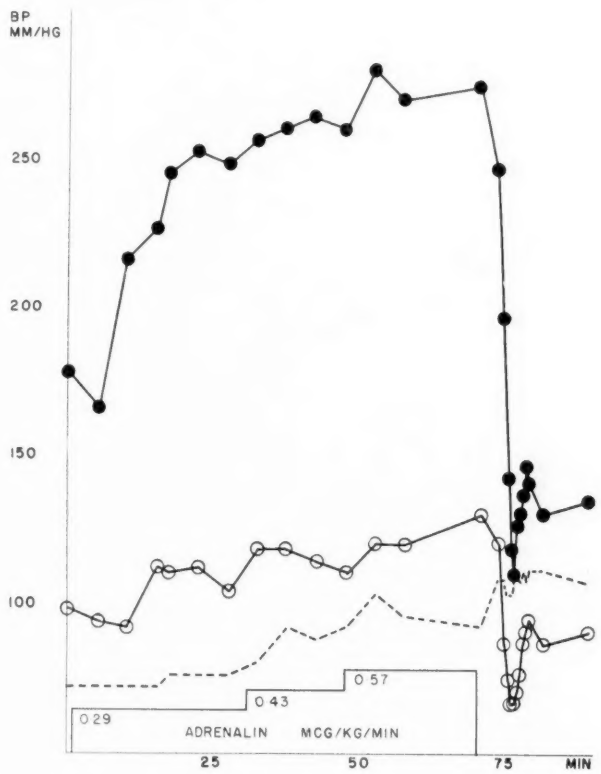

Fig. 1.—The effects of a continuous infusion of adrenalin on pulse rate and blood pressure (Subject J. C.). Solid circles, systolic blood pressure; open circles, diastolic blood pressure; dotted line, pulse rate.

An increase in the rate of administration produced a further rise in systolic and diastolic pressures to a new maximum in one to three minutes. The net result of successive increases in dosage was a steplike curve of pressure change.

In most instances infusion of adrenalin resulted in cardiac acceleration (Table II). The increase in rate usually was most marked at the lowest dosage range where it appeared roughly proportional to the initial pressure. Less than 25 per cent of subjects manifested at any time a fall in rate below the preinfusion value.

TABLE II. CHANGES IN PULSE RATE IN RELATION TO INITIAL DIASTOLIC BLOOD PRESSURE AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

INITIAL DBP RANGE	AVERAGE INITIAL PULSE	CHANGES IN UNIT PULSE RATE (BEATS/MIN./μG/KG./MIN.) AND STANDARD DEVIATION											
		RANGE OF ADRENALIN DOSAGE (μG/KG./MIN.)											
		0.11 – 0.30		0.31 – 0.50		0.51 – 0.70		0.71 – 0.90		0.91 – 1.10		1.11 – 1.30		1.31 – 1.50	
51 – 70	85	7	± 47	13	± 31	14	± 34	−1	± 17	8	± 19	10	± 16	4	± 4
71 – 90	82	32	± 65	32	± 41	25	± 44	22	± 14	18	± 11	12	± 17	17	± 22
91 – 110	80	54	± 55	59	± 45	33	± 24	18	± 21						
111 – 130	82	55	± 67	17	± 31	27	± 21								

Following cessation of infusion, both systolic and diastolic pressures dropped abruptly. A minimum level was reached usually by the tenth and not later than the fifteenth minute after clamping the infusion tubing. These minimum levels were lower than the initial blood pressure levels in all but one instance and were accompanied by cardiac acceleration.

Results of Repeated Infusion.—Observations on the diminished effectiveness of chronic adrenalin administration in allergic disorders, coupled with reports of desensitization phenomena in patients treated for anxiety states by repeated adrenalin administration,[22] prompted a study of the effects of daily infusion.

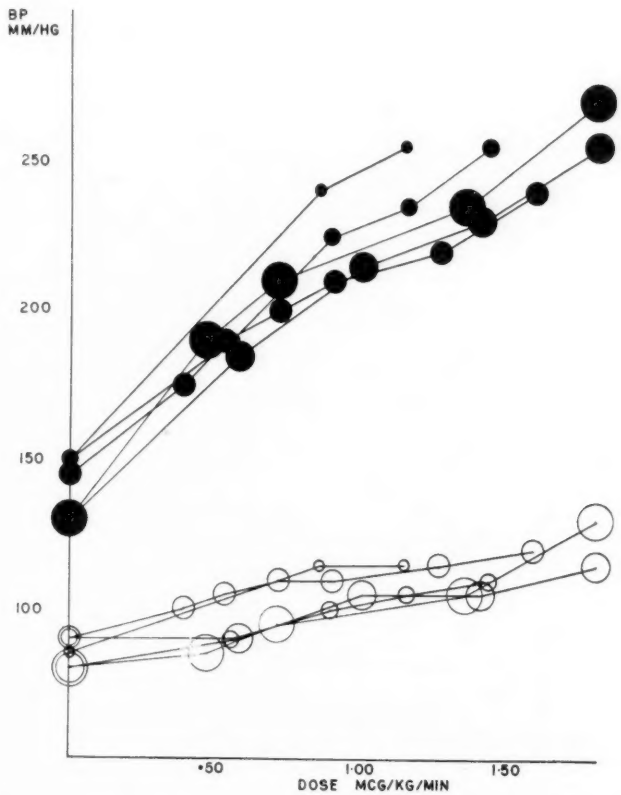

Fig. 2.—The blood pressure responses to daily infusion of adrenalin (Subject J. B.). Size of circles indicates temporal sequence. For clarity, the curves of every second day only are shown. Solid circles, systolic blood pressure; open circles, diastolic blood pressure.

Five subjects were used in this portion of the investigation. One was infused on twelve successive days, a second on ten, another on four, and two on three days. Despite some variation in the initial level of blood pressure from day to day, the slopes of the pressure response curves appeared almost identical within limits of the experimental method, as illustrated in Figs. 2 and 3. For clarity, the curves of alternate days only are presented.

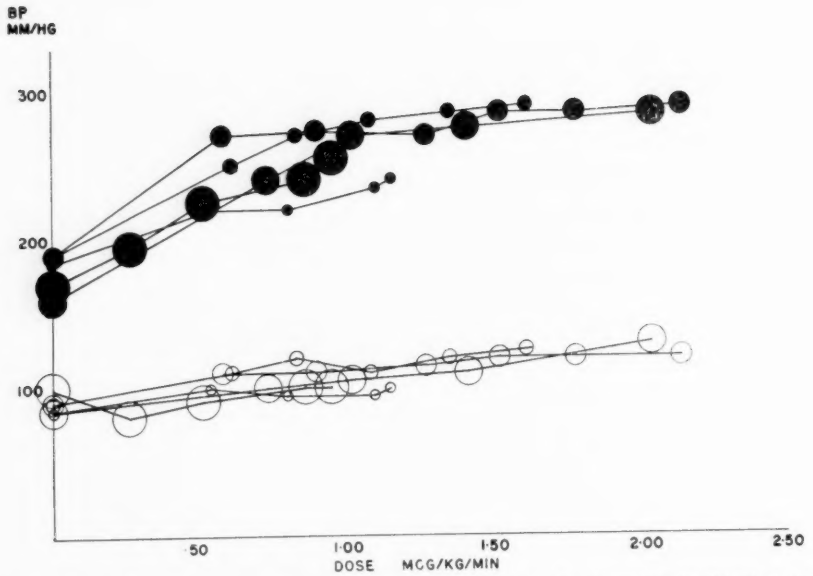

Fig. 3.—The blood pressure responses to daily infusion of adrenalin (Subject J. S.). Size of circles indicates temporal sequence. For clarity, the curves of every second day only are shown. Solid circles, systolic blood pressure; open circles, diastolic blood pressure.

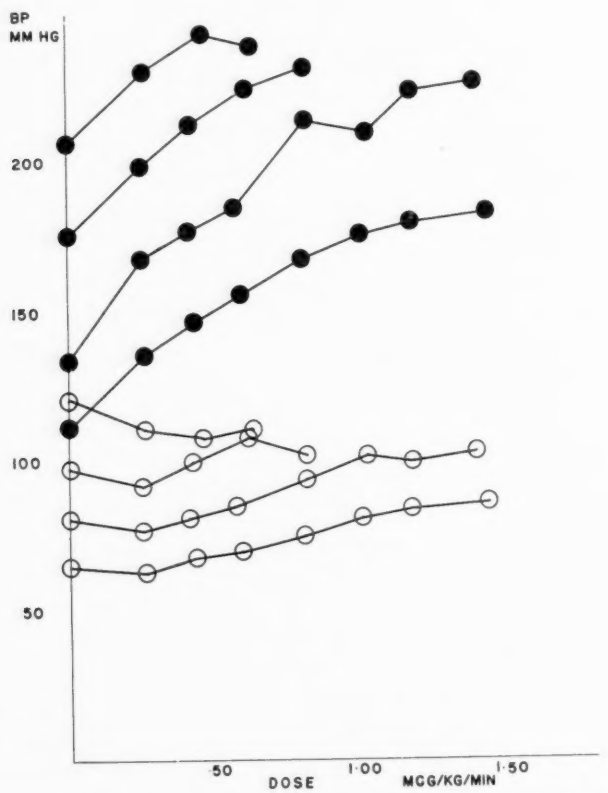

Fig. 4.—Average blood pressure responses to adrenalin in subjects grouped on the basis of initial diastolic pressure. Solid circles, systolic blood pressure; open circles, diastolic blood pressure.

Although objective responses remained reasonably constant, increase in the subjective limit of tolerance was marked. The patient infused on twelve days accepted a dose on the last day which was ten times larger than that tolerated on the first day.

Net Pressure Changes in Relation to Preinfusion Pressure.—The action of adrenalin on both systolic and diastolic pressures was characterized by a wide range of variation between individuals in the same group.

A mean rise in systolic pressure in all groups at all levels of dosage was produced, as illustrated in Fig. 4. The pressure curves tended to parallel one another despite the differences in initial tension.

At the lowest level of dosage a mean fall in diastolic blood pressure was observed which was more marked the higher the initial pressure. Succeeding increments in dosage produced a rise in pressure at a rate roughly parallel in the four pressure groups.

The diastolic fall at low adrenalin dosages in the face of an increase in pulse rate suggested that systolic elevation was achieved through cardiac stimulation which overbalanced a compensatory drop in peripheral resistance. Such a drop in resistance might have resulted from moderator nerve activity alone or in conjunction with a direct dilator action of adrenalin. Whatever the nature of the compensatory mechanism, its value was limited to much the same extent in all groups as evidenced by the transition to diastolic elevation at dosages in excess of 0.30 μg per kilogram per minute.

Unit Pressure Changes in Relation to Preinfusion Pressure.—The rise in systolic pressure per microgram of adrenalin tended to correlate both with increase in pulse rate and with preinfusion systolic pressure over a restricted range of initial tensions, the upper limit of which lay between 180 and 200 mm. Hg. However, when the unit systolic changes over the entire range were compared on a group basis (Table III, Fig. 5), the differences in mean response between groups did not prove significant. The t values for the differences ranged from 0.45 to 1.45.

TABLE III. CHANGES IN BLOOD PRESSURE IN RELATION TO INITIAL DIASTOLIC BLOOD PRESSURE AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

INITIAL DPB RANGE	AVERAGE INITIAL SBP	A. CHANGES IN UNIT SYSTOLIC BLOOD PRESSURE (MM. HG/μG/KG./MIN.) AND STANDARD DEVIATION — RANGE OF ADRENALIN DOSAGE (μG/KG./MIN.)						
		0.11-0.30	0.31-0.50	0.51-0.70	0.71-0.90	0.91-1.10	1.11-1.30	1.31-1.50
51-70	112	91 ± 53	81 ± 36	76 ± 31	70 ± 20	63 ± 14	58 ± 13	50 ± 9
71-90	134	137 ± 132	103 ± 65	90 ± 38	99 ± 33	74 ± 14	77 ± 13	67 ± 1
91-110	176	91 ± 86	93 ± 93	81 ± 48	69 ± 12			
111-130	207	89 ± 67	79 ± 46	54 ± 34				
Average		106 ± 96	91 ± 62	80 ± 38	80 ± 17	69 ± 14	69 ± 16	57 ± 12

INITIAL DPB RANGE	AVERAGE INITIAL DBP	B. CHANGES IN UNIT DIASTOLIC BLOOD PRESSURE (MM. HG/μG/KG./MIN.) AND STANDARD DEVIATION — RANGE OF ADRENALIN DOSAGE (μG/KG./MIN.)						
		0.11-0.30	0.31-0.50	0.51-0.70	0.71-0.90	0.91-1.10	1.11-1.30	1.31-1.50
51-70	65	−11 ± 36	1 ± 23	9 ± 17	13 ± 12	16 ± 8	16 ± 7	15 ± 4
71-90	81	−14 ± 33	0 ± 28	8 ± 7	16 ± 10	21 ± 5	17 ± 9	16 ± 1
91-110	98	−23 ± 34	5 ± 34	15 ± 24	1 ± 22			
111-130	121	−40 ± 28	−28 ± 26	−15 ± 21				
Average		−19 ± 33	−2 ± 29	9 ± 19	12 ± 13	17 ± 7	16 ± 8	15 ± 3

When the degree of activity at various dosages was computed, it became evident that adrenalin was decreasingly effective in its ability to elevate the systolic pressure as the rate of administration was increased. The over-all unit response was halved with a sixfold rise in dose, while the maximum response, which averaged 106 mm. Hg per microgram, was observed at the lowest dosage range.

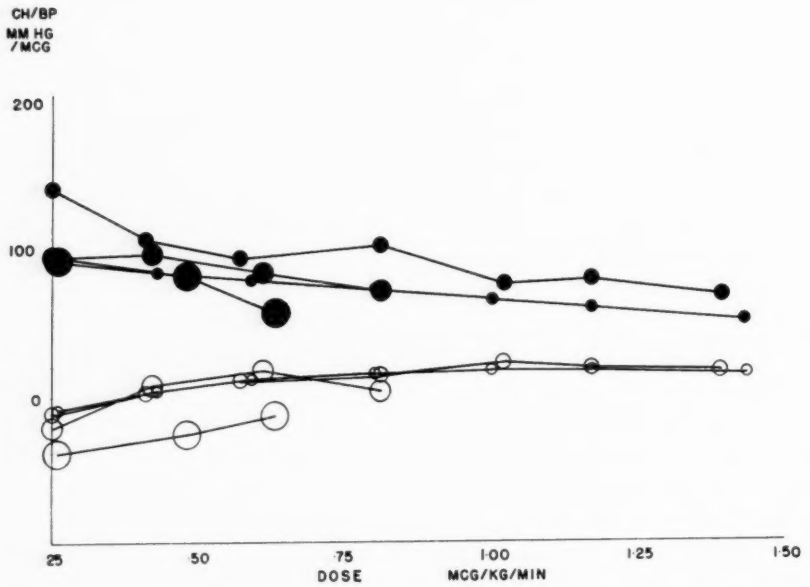

Fig. 5.—Average unit blood pressure responses to adrenalin in subjects grouped on the basis of initial diastolic pressure. Size of circles indicates the relative magnitude of the initial blood pressure range. Solid circles, systolic blood pressure; open circles, diastolic blood pressure.

Comparison of changes in unit diastolic pressure (Table III, b, Fig. 5) demonstrated a fall at the lowest rate of administration which appeared inversely proportional to the initial pressure, but the coefficient of correlation (–0.229) was not significant. Succeeding increases in rate resulted in a rise to a maximum value of 17 mm. Hg per microgram at an average dose of 1.0 μg per kilogram per minute, a value which showed little further change at higher dosage levels where a comparison between groups was possible.

After allowance was made for the wide range of individual variation, there was evident a common pattern of reaction characterized by pressure changes similar in directions and magnitudes in each of the four pressure groups but different in the pressure base lines from which projected.

Effects on the Systolic-Diastolic Pressure Relationship.—The similarity of the unit pressure changes in each group suggested that adrenalin was modifying some relationship between systolic and diastolic pressure which was largely independent of the absolute height of the pressure. To explore this possibility, calculations were made of the correlation coefficient between systolic and diastolic pressures initially and at each dosage level. The value of the coefficient

(Table IV) averaged 0.80, which indicated the probability that the two pressures were related by a fairly constant factor. The magnitude of this factor was estimated by determining the systolic-diastolic ratios at each rate of administration.

TABLE IV. CORRELATION BETWEEN SYSTOLIC AND DIASTOLIC BLOOD PRESSURES AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

DOSAGE RANGE (μG/KG./MIN.)	CORRELATION COEFFICIENT
0	0.817
0.11-0.30	0.754
0.31-0.50	0.795
0.51-0.70	0.781
0.71-0.90	0.814
0.91-1.10	0.718
1.11-1.30	0.885
1.31-1.50	0.829
Average	0.800

The preinfusion ratio averaged 1.7 ± 0.27 and did not vary materially among the four pressure groups. The size of this ratio was somewhat surprising, for its value at normal pressures is usually stated to approximate 1.5,[23a, p. 127, 23b] while the increased peripheral resistance in hypertensive subjects is expected to exaggerate the diastolic pressure[24] and yield a ratio lower than normal. However, a calculation of this ratio from data reported by Steele[25] for a group of thirty-nine patients whose blood pressures ranged from 94 mm. Hg systolic, 60 mm. diastolic to 262 mm. systolic, 164 mm. diastolic gave an average value of 1.72 ± 0.10. Similar calculations were made from Goldring and

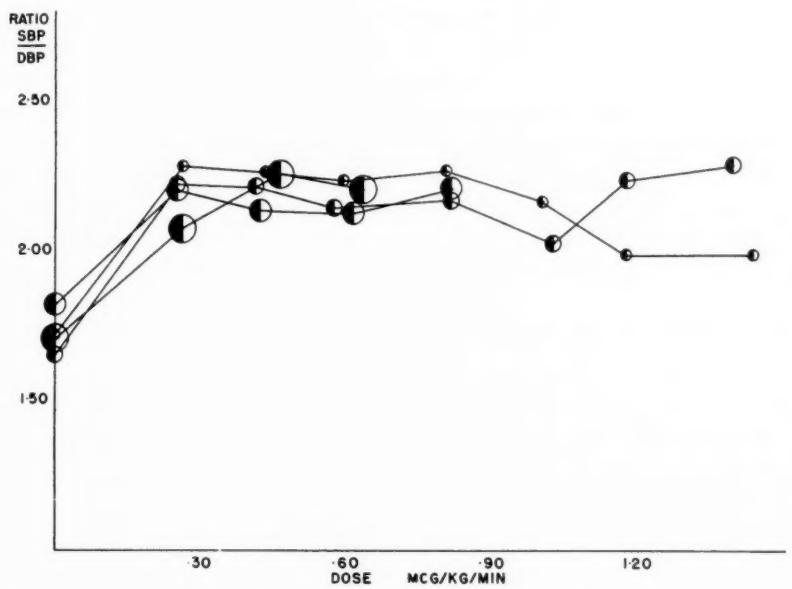

Fig. 6.—Average change in systolic-diastolic ratio produced by adrenalin in subjects grouped on the basis of initial diastolic pressure. Size of circles indicates the relative magnitude of the initial blood pressure range.

Chasis' data[15, pp. 20-21] relative to fifty-six patients with mild to severe hypertension. The ratio of this latter group averaged 1.59 ± 0.19 for both minimum and maximum recorded pressures.

At the lowest rate of adrenalin administration, the systolic-diastolic ratio in the present series increased to an average value of 2.21 ± 0.38. This new value was also similar in all pressure groups and tended to remain constant with succeeding increments in dosage. These relations are illustrated in Fig. 6 and their variability indicated in Table V. The t value (9.78) for the difference in ratio before and during infusion at the lowest dose range indicated the change to be highly significant.

TABLE V. SYSTOLIC-DIASTOLIC PRESSURE RATIO IN RELATION TO INITIAL DIASTOLIC BLOOD PRESSURE AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

INITIAL DBP RANGE	RANGE OF ADRENALIN DOSAGE (μG/KG./MIN.)							
	0	0.11 – 0.30	0.31 – 0.50	0.51 – 0.70	0.71 – 0.90	0.91 – 1.10	1.11 – 1.30	1.31 – 1.50
51– 70	1.72 ± 0.21	2.28 ± 0.25	2.26 ± 0.31	2.23 ± 0.28	2.26 ± 0.31	2.16 ± 0.25	1.98 ± 0.32	1.98 ± 0.30
71– 90	1.65 ± 0.26	2.22 ± 0.53	2.21 ± 0.47	2.14 ± 0.38	2.16 ± 0.13	2.02 ± 0.50	2.23 ± 0.15	2.28 ± 0.20
91–110	1.82 ± 0.34	2.20 ± 0.35	2.13 ± 0.38	2.12 ± 0.30	2.20 ± 0.39			
111–130	1.71 ± 0.07	2.07 ± 0.07	2.25 ± 0.13	2.20 ± 0.12				
Average:	1.73 ± 0.27	2.21 ± 0.38	2.21 ± 0.37	2.17 ± 0.31	2.22 ± 0.26	2.10 ± 0.30	2.12 ± 0.24	2.10 ± 0.27

It would appear likely that this increase in systolic-diastolic ratio reflects a readjustment in the relation of peripheral resistance to cardiac output which results from adrenalin action and is relatively independent of the initial pressure.

Postinfusion Fall in Pressure.—When blood pressure is raised by application of a pressor agent which can be quickly destroyed, it is anticipated that the pressure will return at least to the initial level when the pressor agent is no longer supplied. In order to avoid such spurious correlations, all calculations involving the fall which followed adrenalin infusion were based on the amount by which the blood pressure dropped below the preinfusion level.

Computed on this basis, the postinfusion fall in both systolic and diastolic blood pressures was found to correlate more closely with the height of the initial blood pressure than with rise in pressure, maximum pressure, dose, or maximum unit change in blood pressure, as shown in Table VI.

TABLE VI. CORRELATION BETWEEN FALL IN POSTINFUSION BLOOD PRESSURE BELOW INITIAL LEVEL AND VARIOUS MODIFYING INFLUENCES

FALL IN BLOOD PRESSURE AND:	COEFFICIENT OF CORRELATION (SYSTOLIC PRESSURE)	COEFFICIENT OF CORRELATION (DIASTOLIC PRESSURE)
Initial pressure	–0.598	–0.491
Maximum pressure	–0.500	–0.307
Maximum change in pressure	0.032	0.134
Dose	–0.389	–0.117
Change in unit pressure	0.079	0.239

In general, the higher the patient's initial pressure, the further did the pressure drop below the initial level when the infusion was stopped. The extent of this relation is illustrated in Figs. 7 and 8. In a number of instances the

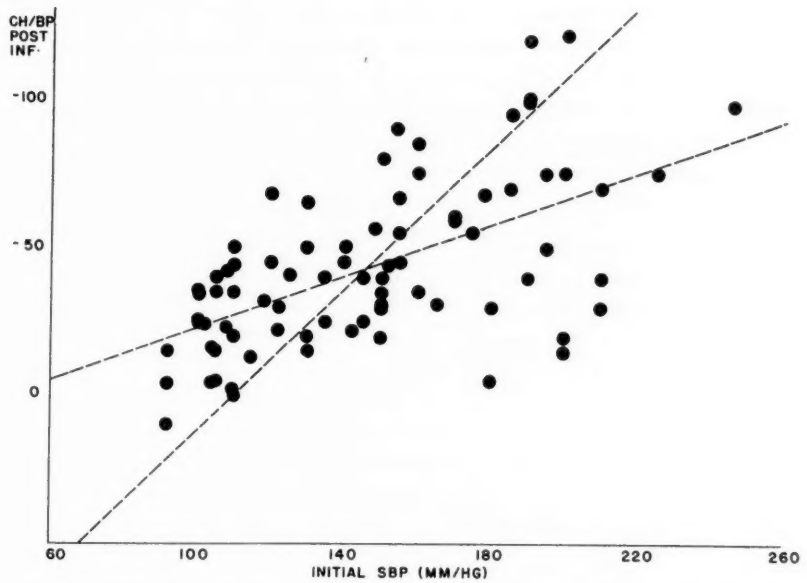

Fig. 7.—The relation between initial systolic pressure and the postinfusion fall below initial pressure. The lines of regression from the X and Y axes are indicated.

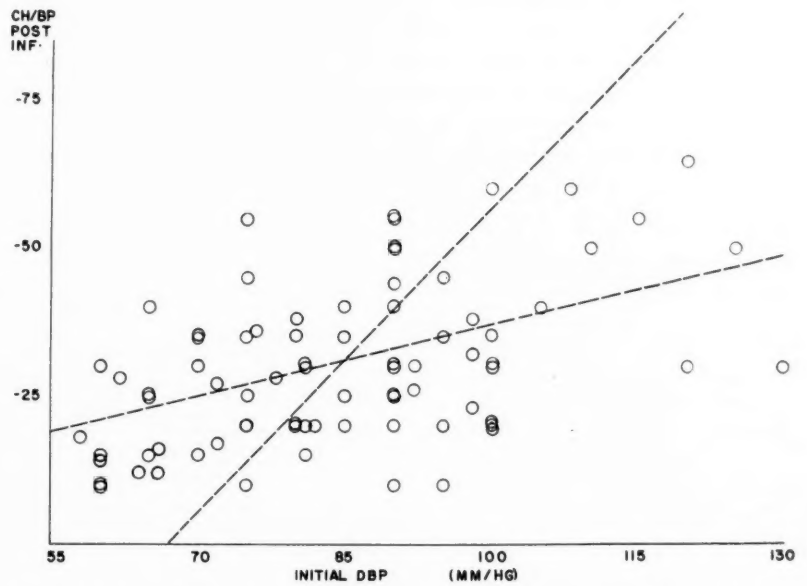

Fig. 8.—The relation between initial diastolic pressure and the postinfusion fall below initial pressure. The lines of regression from the X and Y axes are indicated.

minimum pressures came within the range usually associated with shock. Except for tachycardia, however, no other signs of shock were noted. The subjects remarked about a sensation of heat and manifested a widespread vasodilatation by a generalized flushing of the skin. Tilting during the stage of blood pres-

sure depression was followed by considerable acceleration of the pulse rate and, in one instance, by syncope. The reactions to tilting were similar to the ones which follow bleeding.[26]

The cause of the postinfusion drop in tension to subnormal levels is questionable. The depression of pressure in association with evidences of vasodilatation suggests a temporary persistence of compensatory vasodepressor activity, either nervous or humoral. The presence of postural vasomotor reflexes makes it unlikely that adrenalin blockage of sympathetic ganglia[27] is the responsible factor. The tendency of the hypertensive subjects to display a more marked postinfusion pressure depression accords with the results of carotid sinus stimulation.[1]

The possible role of blood volume changes is considered in connection with blood concentration.

Alterations in Hemoconcentration.—Two potential mechanisms exist by which adrenalin might alter blood concentrations. The first involves splenic contraction followed by extrusion of sequestrated red cells and an increase in their percentage in the circulating blood.[23a, p. 54, 28] The second possibility implicates leakage of plasma through capillaries made more permeable by stagnant anoxia following prolonged vasoconstriction.[29]

These possibilities were studied by using the hematocrit value as an indicator of blood concentration. The results of determinations made during and after infusion were compared with the initial values. No significant changes were demonstrated at either stage within the range of dosage used (Table VII).

The constancy of the hematocrit value makes it unlikely that the postinfusion blood pressure depression displayed by the subjects of this investigation was due to a reduction in plasma volume comparable to that produced in dogs by Freeman and co-workers through administration of adrenalin in shock dosages.[29]

TABLE VII. EFFECTS OF ADRENALIN INFUSION ON BLOOD CONCENTRATION

TIME	HEMATOCRIT VALUE (VOL. %)
a. Average Values	
(1) Before infusion	39
(2) During infusion	40
(3) After infusion	39
b. Standard Deviations of the Differences in Mean Hematocrit Value During and After Infusion as Compared with Preinfusion Average	
d (1) and (2)	1 ± 2.9
d (1) and (3)	0 ± 3.7

SUMMARY AND CONCLUSIONS

Administration of eighty continuous infusions of adrenalin to fifty-one subjects evoked a common pattern of systolic and diastolic response of all ranges of initial pressure.

The correlation between the height of the initial pressure and the depth of the subsequent postinfusion depression provides additional data that the vasodilator capacity of the hypertensive individual is enhanced rather than diminished.

The sum of these responses to adrenalin together with the extent of the fall produced by carotid sinus stimulation, spinal anesthesia, pyrogens, and the tetraethylammonium ion indicates that "fixed hypertension" in the sense of a pressure elevation incapable of material reduction is a concept of questionable reality. On the contrary, the accumulated evidence demonstrates the availability of compensatory mechanisms in the hypertensive subject and the capacity of the vascular apparatus to respond effectively when these mechanisms are properly stimulated.

The existence of pressure elevation in the presence of potentially effective restorative mechanisms suggests that sustained hypertension is associated, like fever, with an upward shift in the base line from which the homeostatic mechanisms are operative. The problem ahead is to determine the factor or factors responsible for the elevation of the pressure base line to this new level.

REFERENCES

1. Weiss, S., and Baker, J. P.: Carotid Sinus Reflex in Health and Disease; Its Role in Causation of Fainting and Convulsions, Medicine **12**: 297-354, 1933.
2. Moore, F. H., and Allen, E. V.: Vascular Clinics; Carotid Sinus in Hypertension, Proc. Staff Meet., Mayo Clin. **13**: 747-751, 1938.
3. Thomas, C. B.: Experimental Hypertension From Section of Moderator Nerves; Relationship of Acute Pressor Response to Development and Course of Chronic Hypertension, Bull. Johns Hopkins Hosp. **74**: 335-377, 1944.
4. Allen, E. V., Lundy, J. S., and Adson, A. W.: Preoperative Prediction of Effects of Blood Pressure on Neurosurgical Treatment of Hypertension, Proc. Staff Meet., Mayo Clin. **11**: 401-406, 1936.
5. Gregory, R., and Levin, W. C.: Studies on Hypertension; Effect of High Spinal Anesthesia on Blood Pressure of Patients With Hypertension and Far-Advanced Renal Disease—Its Possible Relationship to Pathogenesis of Hypertension, J. Lab. & Clin. Med. **30**: 1037-1043, 1945.
6. Chasis, H., Goldring, W., and Smith, H. W.: Reduction of Blood Pressure Associated With Pyrogenic Reaction in Hypertensive Subjects, J. Clin. Investigation **21**: 369-376, 1942.
7. Berry, Robert L., Campbell, Kenneth M., Lyons, R. H., Moe, G. K., and Sutler, M. R.: The Use of Tetraethylammonium in Peripheral Vascular Disease and Causalgic States, Surgery 20: 525-535, 1946.
8. Hines, E. A., Jr., and Brown, G. E.: Standard Test for Measuring Variability of Blood Pressure: Its Significance as Index of Pre-Hypertensive State, Ann. Int. Med. **7**: 209-217, 1933.
9. Pickering, G. W., and Kissin, M.: Effects of Adrenaline and of Cold on Blood Pressure in Human Hypertension, Clin. Sc. **2**: 201-207, 1936.
10. Miller, J. H., and Bruger, M.: Cold-Pressor Reaction in Normal Subjects and in Patients With Primary (Essential) and Secondary (Renal) Hypertension, Am. Heart J. **18**: 329-333, 1939.
11. Alam, M., and Smirk, F. H.: Blood Pressure Raising Reflexes in Health, Essential Hypertension, and Renal Hypertension, Clin. Sc. **3**: 359-266, 1938.
12. Chesley, L. C., and Chesley, E. R.: Cold Pressor Test in Pregnancy, Surg., Gynec. & Obst. **69**: 436-440, 1939.
13. Wellen, I.: Quoted by Goldring and Chasis.
14. Russek, H. I., and Zohman, B. L.: Influence of Age Upon Blood Pressure Response to Cold-Pressor Test, Am. Heart J. **29**: 113-119, 1945.
15. Goldring, William, and Chasis, Herbert: Hypertension and Hypertensive Disease, New York, 1944, The Commonwealth Fund.
16. Ayman, D., and Goldshine, H. D.: Breath-Holding Test; Simple Standard Stimulus of Blood Pressure, Arch. Int. Med. **63**: 899-906, 1939.
17. Feldt, R. H., and Wenstrand, D. E. W.: Cold Pressor and Breath-Holding Test; Analysis of Results in 200 Subjects, Arch. Int. Med. **67**: 1157-1161, 1941.
18. Clough, P. W.: A Study of the Cardiovascular Reaction to Epinephrine, Bull. Johns Hopkins Hosp. **31**: 266, 1920.
19. Hougardy, A.: De l'action hypertensive de l'adrénaline chez le chien hypotendu par décapsulation, Compt. rend. Soc. de biol. **113**: 1195, 1933.

20. Koehler, A. E., Marsh, N., and Hill, E.: The Effect of Epinephrine Injected Intravenously at a Constant Rate in Normal and Hypertensive Cases, J. Biol. Chem. **119:** LIX, 1937.
21. Green, D. M.: Pheochromocytoma and Chronic Hypertension, J. A. M. A. **131:** 1260-1265, 1946.
22. Cameron, D. E.: Adrenalin Administration in Persistent Anxiety States, Am. J. Med. Sc. **210:** 281, 1945.
23. (a) Best, C. H., and Taylor, N. B.: The Physiological Basis of Medical Practice, ed. 4, Baltimore, 1945, The Williams and Wilkins Company.
(b) Herrmann, George R.: Synopsis of Diseases of the Heart and Arteries, ed. 3, St. Louis, 1944, The C. V. Mosby Company, p. 153.
24. Wiggers, C. J.: Physiology in Health and Disease, ed. 4, Philadelphia, 1944, Lea and Febiger, p. 644.
25. Steele, J. M.: Comparison of Simultaneous Indirect (Auscultatory) and Direct (Intra-arterial) Measurements of Arterial Pressure in Man, J. Mt. Sinai Hosp. **8:** 1042, 1942.
26. Green, D. M., and Metheny, D.: The Estimation of Acute Blood Loss by the Tilt Test, Surg., Gynec. & Obst. **84:** 1045-1050, 1947.
27. (a) Marrazzi, A. S.: Adrenergic Inhibition at Sympathetic Synapses, Am. J. Physiol. **127:** 738, 1939.
(b) Bulbring, E., and Burn, J. H.: An Action of Adrenalin on Transmission in Sympathetic Ganglia Which May Play a Part in Shock, J. Physiol. **101:** 289-303, 1942.
28. Watson, C. J., and Paine, J. R.: A Study of the Splenic Venous Blood With Particular Reference to the Hematocrit Percentage and Hemoglobin Concentration of the Erythrocytes Before and After Splenic Arterial Injections of Adrenalin, Am. J. M. Sc. **205:** 493, 1943.
29. Freeman, N. E., Freedman, H., and Miller, C. C.: The Production of Shock by the Prolonged Continuous Injection of Adrenalin in Unanesthetized Dogs, Am. J. Physiol. **131:** 545-553, 1941.

THE EFFECT OF OPERATION AND ILLNESS ON CLOT RETRACTION: DESCRIPTION OF A NEW METHOD

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LAMPERT,[1] in 1935, indicated the possible role which accelerated clot retraction may play in phlebothrombosis and pulmonary embolism. Hirschboeck and Coffey[2] have recently demonstrated that a shortening of the clot retraction time occurs in patients who have had a recent pulmonary embolism. Because of the difficulty in observing the end point in the usual blood coagulation and clot retracting tests, it was decided to search for a more simple and decisive method of observation. The coagulation-retraction test herein described has been found to be more useful than the older methods and was used in obtaining the data for this report.

Clot retraction is dependent upon thrombocytes, surface forces, erythrocyte mass, and qualitative and quantitative variations in fibrin. Other factors as yet unknown, also may influence the process. It is common knowledge that clot retraction is greatly delayed or does not occur when thrombocytopenia is present. Thrombocytosis is, on the other hand, associated with rapid clot retraction.[2]

It is not known how the thrombocytes actually participate in the phenomenon. Tocantins' explanation that the agglutinated, disintegrating thrombocytes, which collect and adhere to the fibrin strands, fuse with adjacent thrombocyte masses, thus causing the gradual pulling together of the fibrin mesh, has been widely accepted.[3]

The surface with which the clot is in contact greatly influences the retraction. A collodion surface is completely inhibitory.[4] Paraffin, methyl methacrylate, and other water repellant materials which inhibit coagulation also more or less inhibit clot retraction. The surface of the vascular endothelium apparently exerts a similar effect. These surfaces may act because of their ability to inhibit platelet agglutination or because the fibrin becomes more firmly attached to the surface.[5]

Erythrocytes influence clot retraction in two ways. First, when the concentration, as measured by the hematocrit, is abnormally great, for example 80 per cent (a value frequently seen in congenital heart disease with chronic anoxemia), clot retraction does not occur. The mass of erythrocytes, which, incidentally, has an entirely passive role in coagulation, limits the degree of retraction by the volume which it occupies in the clot. Second, when the erythrocyte mass is large, the plasma volume, and hence the total amount of fibrinogen, is proportionately small. The opposite condition prevails in anemia. Here the small erythrocyte mass interferes less with clot retraction and the clot

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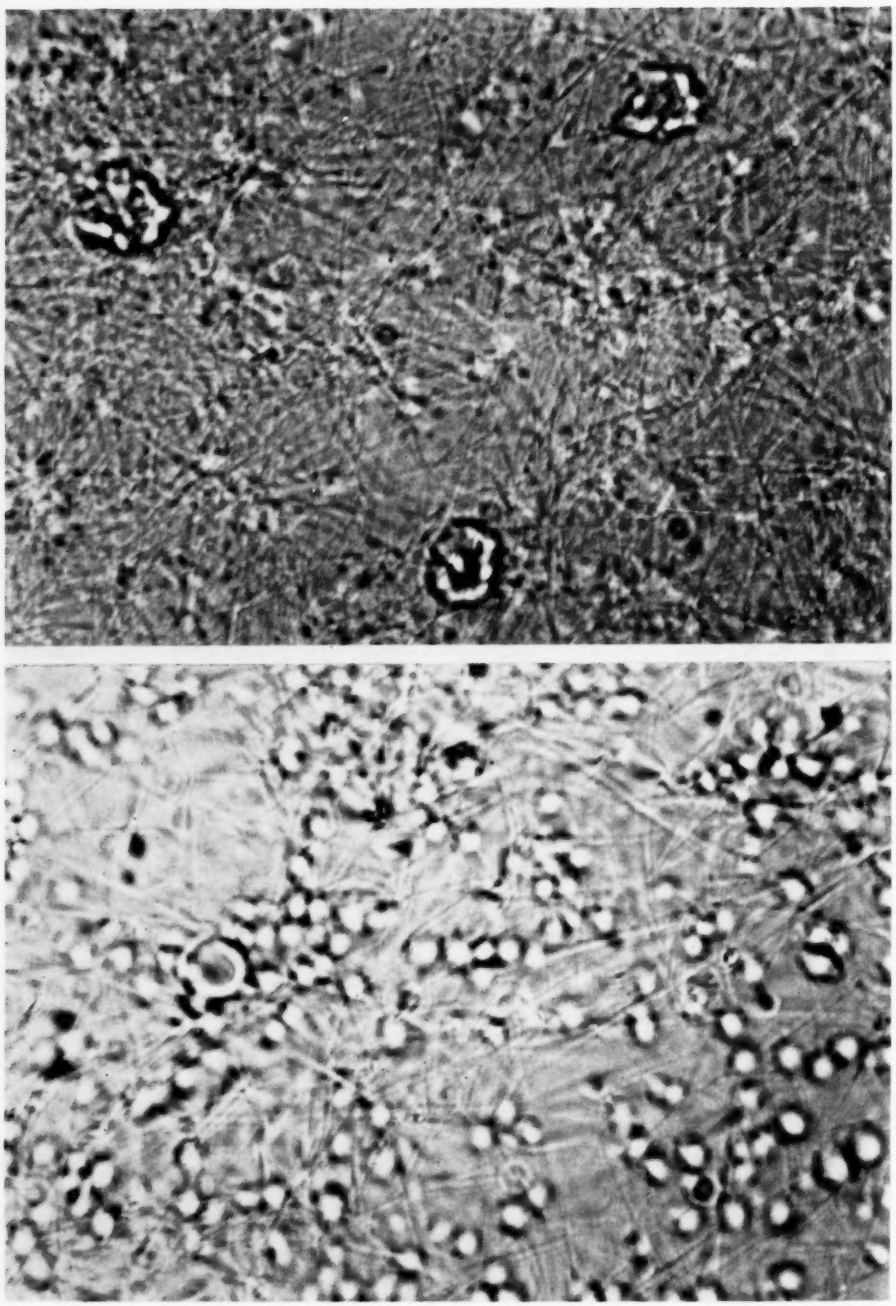

Fig. 1.—The effect of surface on platelet disintegration and fibrin formation. The upper photograph was taken one hour after plasma from freshly drawn, centrifuged, human blood was placed on a glass surface (microscope slide and cover slip). Note the disintegrating platelets and the numerous delicate fibrin strands. The large bodies are erythrocytes. The lower photograph demonstrates the effect of a collodion surface on the same specimen of plasma. The platelets are intact and highly refractile and the fibrin strands are heavier and less numerous.

contains more fibrin because of the relative or even absolute hyperfibrinogenemia. Thus anemic blood exhibits a tendency toward more rapid and extensive clot retraction. A fibrinogen fraction which greatly accelerates erythrocyte sedimentation, and is converted into rapidly retracting fibrin (contractinogen),[6] may be present. These qualitative and quantitative increases in fibrin which occur during illness are responsible not only for increasing the rate of erythrocyte sedimentation but also for producing denser and more retractile clots.

A study of the thrombotic diathesis or, as some prefer to say, hypercoagulability, must include more than the mere measurement of coagulation time. A method which will measure indirectly the combined effect of anemia, thrombocytosis, and hyperfibrinogenemia, in addition to any other factor which may express itself in a shortened coagulation time, should be valuable. Earlier observations of the clot retraction time form the basis for much of the present work.[2] A new method using capillary blood has been devised. It has the advantages of eliminating venipuncture and of having a more precise end point.

METHOD FOR DETERMINING THE CLOT RETRACTION TIME IN CASTOR OIL

The skin of the finger tip is cleansed with alcohol and a puncture 3 mm. deep is made with a No. 11 Bard-Parker blade with a cork guard set 3 mm. from the tip. The time is recorded when the skin puncture is made. Two samples of blood (20 c.mm. each) are drawn as quickly as possible into Sahli hemoglobinometer pipettes. The same pipette may be used for each sample if the blood flows freely and the aspiration is done quickly. Each sample of blood is suspended in a separate test tube filled with castor oil, U. S. P., in the following manner. The blood is expelled from the pipette as a large single drop which is "planted" on the center of the oil surface by touching the tip of the pipette to the oil. The drop will settle into the oil and hang by surface attraction to the meniscus. Castor oil was selected because its specific gravity at room temperature is almost the same as the specific gravity of blood. The tubes are stoppered to prevent evaporation. This procedure should not take longer than twenty seconds. Two samples are used for purposes of control. If the clot retraction time is not equal or within three minutes of being equal in both tubes, the test is repeated. This is seldom necessary. The tubes are placed in a rack at room temperature and are observed at the end of ten-, fifteen-, and twenty-minute periods. The end point of coagulation is not determined. The beginning of clot retraction is established when a visible dimpling of the clot surface with extrusion of a tiny droplet of serum occurs. It is usually readily seen because of the highly refractile oil-blood interface. This is the end point of the test. The droplet of serum enlarges until clot retraction is complete (Figs. 2 and 3). For investigative purposes, the preparations were observed continually.

It was found that normal individuals have a clot retraction time of 20 minutes or longer, the average being 33.1 minutes. Clot retraction times longer than 50 minutes were observed only in thrombocytopenia and erythremia and in persons with a coagulation defect. The clot retraction time may fluctuate during the course of the day in some individuals. The moving of patients in

Fig. 1.—Series of photographs showing the development of a bud of serum from a drop of blood allowed to coagulate and retract in castor oil. The time interval between each picture is approximately one-half minute.

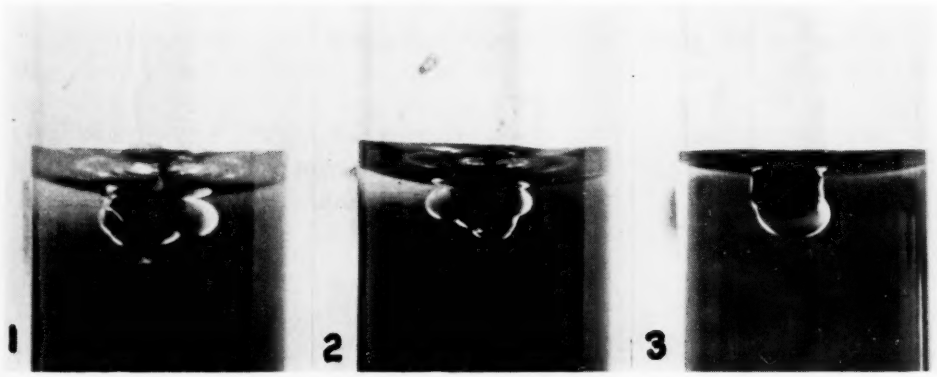

Fig. 3.—Three types of serum buds observed when a drop of capillary blood coagulates and retracts in castor oil.

and out of bed has been associated with fluctuations of more than 8 minutes in some of the more senile individuals. It is thought that this may be due to minor variations in thrombocyte levels. The clot retraction time is shorter at 37° C. Variations in room temperature have not appreciably influenced the results. Room temperature was selected for the test because the drops of blood frequently fall in the oil at 37° C.

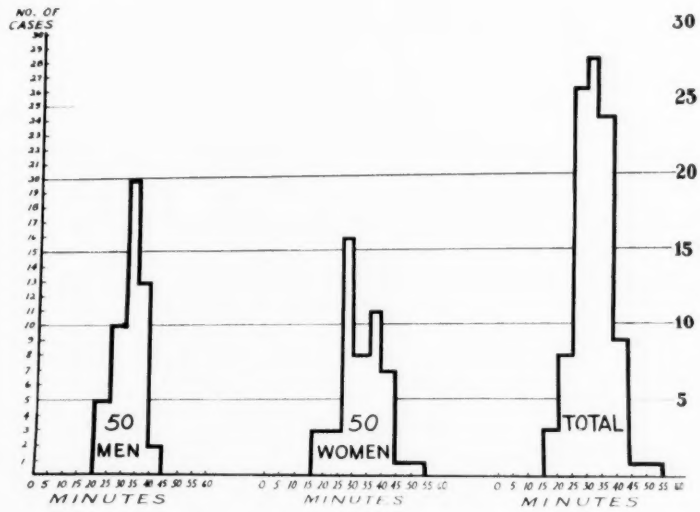

Fig. 4.—Normal variations in the coagulation-retraction time in a series of one hundred normal individuals.

The clot retraction time was determined in a series of fifty normal men and fifty normal women. Hospital attendants, nurses, and medical students were used as subjects. The mean clot retraction time for men was 32.4 minutes and for women was 33.8 minutes, or 33.1 minutes for the group as a whole (Fig. 4). It was noticed that active menstruation slightly shortened the clot retraction time. Eleven women had a mean clot retraction time of 27.6 minutes during active menstruation and 34.7 minutes during the intermenstrual period.

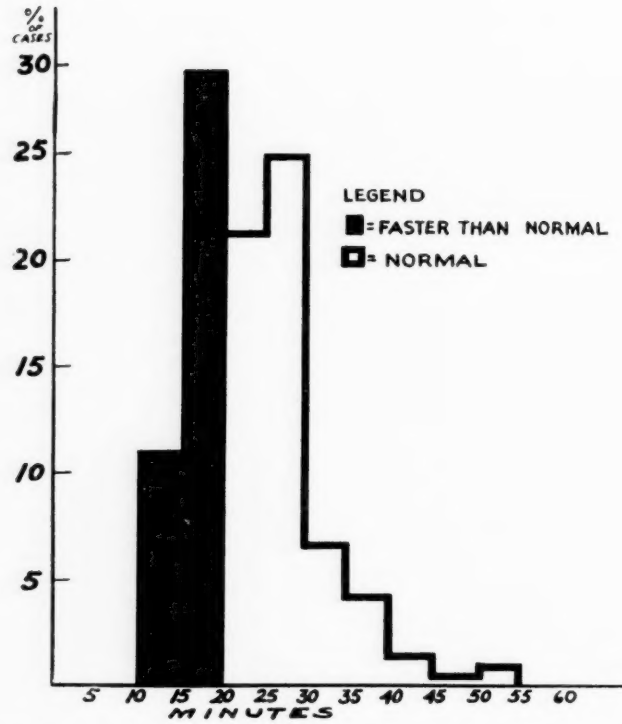

Fig. 5.—The "shift to the left" in coagulation-retraction times observed in three hundred general medical patients.

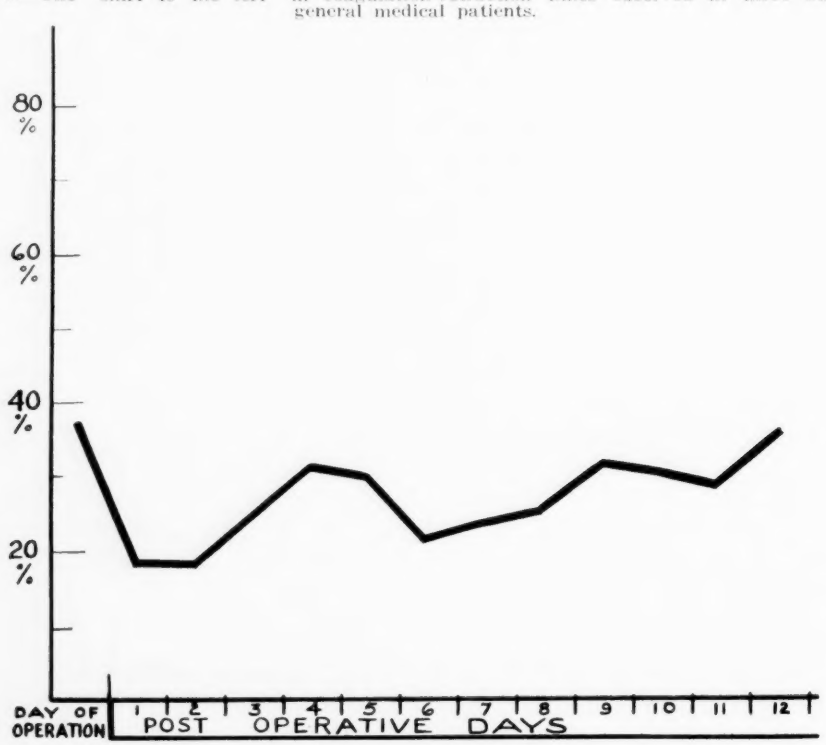

Fig. 6.—Postoperative variations in the incidence of short (pathologic; 20 minutes or less) coagulation-retraction times.

Observations in the general hospital patient population, excluding patients in the first postoperative month, revealed that the mean clot retraction time was shorter than in healthy persons (Fig. 5). The mean clot retraction time for one hundred fifty-two men was 24.9 minutes and for one hundred forty-eight women, 22.6 minutes, or 23.8 minutes for the total group (three hundred). The clot retraction time was less than 20 minutes in 23.7 per cent and less than 15 minutes in 7.6 per cent of the group.

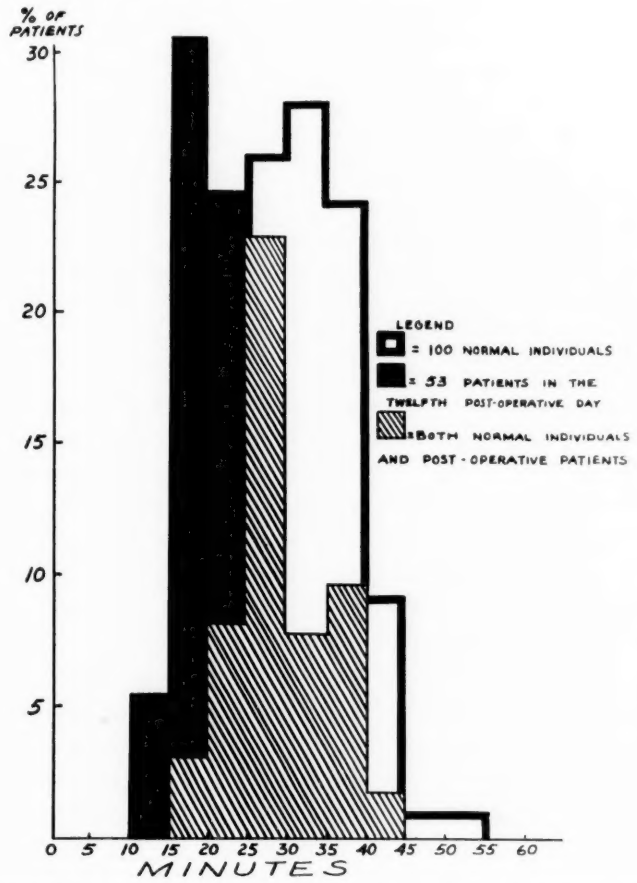

Fig. 7.—A comparison between the coagulation-retraction times of normal and postoperative patients. The cross-ruled area represents the distribution region common to both groups.

One hundred surgical patients were observed during the postoperative period. The majority had undergone major abdominal surgery. Fig. 6 shows the tendency of the clot retraction time to fluctuate during the postoperative period. Of the tests performed on the day of surgery, 33.5 per cent were faster than normal. This may be explained by the hyperfibrinogenemia resulting from the stimulus of surgical trauma and perhaps dehydration. The percentage of abnormally short clot retraction times was also high on the third, fourth, and fifth, and on the ninth and subsequent postoperative days. Fig. 7 is a comparison between the clot retraction time of fifty-three patients in their twelfth post-

operative day with one hundred normal individuals. Seventy-six of the one hundred postoperative patients had an abnormally short clot retraction time on one or more days during the period. In thirty of the patients the clot retraction time was shorter than 15 minutes on one or more days.

DISCUSSION

In the past, great emphasis has been placed on vascular endothelial trauma and delayed circulation time as factors in the cause of phlebothrombosis and pulmonary embolism. Since the introduction of anticoagulant therapy more attention has been given to the blood itself. The thrombotic diathesis apart from slowing of circulation time and vascular trauma, is entirely dependent upon hemic factors, namely thrombocytosis, hyperfibrinogenemia, and diminished erythrocyte mass. These factors may enhance coagulation, but their more important effect lies in their creation of a strongly retracting, tough clot which is susceptible to embolic detachment because of its retractility.

One of the patients in the nonsurgical group, a woman who had recently been digitalized for early total myocardial failure, developed pulmonary embolism when her clot retraction time was 11 minutes. The clinical picture was typical and the radiographic examination of the chest and the electrocardiogram sustained the diagnosis. This was the only instance of pulmonary embolism encountered during the entire study. A correlation between the clot retraction time in castor oil and the tendency toward pulmonary embolism is implied in this case. Likewise, the shortening of the clot retraction time in bedridden patients may help to explain the tendency toward pulmonary embolism prevalent in that group. If we can assume that patients with short clot retraction times are more susceptible to thrombosis and embolism, it may be desirable to treat those persons prophylactically with anticoagulants.

The clot retraction test is well adapted to the control of heparin therapy and can be used instead of the ordinary coagulation time determinations. It is not reliable during Dicumarol therapy. The test is really a measure of the coagulation time plus the interval between the end of coagulation and the beginning of clot retraction. Heparin, since it prolongs coagulation and in some instances inhibits the postcoagulation phase, will greatly lengthen the time of the test. Dicumarolization frequently prolongs the clot retraction time, but there is no correlation between this effect and the prothrombin time.

SUMMARY

A test for measuring the blood clot retraction time in castor oil is described.

The clot retraction time is considerably shorter than normal in certain patients who are in the postoperative period and in almost one-fourth of all general medical patients.

The test measures factors which are part of the hemic phase of the thrombotic diathesis.

REFERENCES

1. Lampert, H.: Medizinische Kolloidlehre, Dresden, 1935, Theodor Steinkopff.
2. Hirschboeck, J. S., and Coffey, W. L., Jr.: Clot Retraction in Thrombophlebitis and Pulmonary Embolism, Am. J. M. Sc. **205**: 727-730, 1943.
3. Tocantins, L. M.: Platelets and the Structure and Physical Properties of Blood Clots, Am. J. Physiol. **114**: 709-715, 1936; Platelets and the Spontaneous Syneresis of Blood Clots, ibid. **110**: 278-286, 1934.
4. Hirschboeck, J. S.: Delayed Blood Coagulation and Absence of Clot Retraction in Collodion Lined Vessels, Proc. Soc. Exper. Biol. & Med. **45**: 122-124, 1940.
5. Quick, A. J.: Personal communication.
6. Morrison Ira R.: Qualitative Changes in Fibrinogen Which Influence Erythrocyte Sedimentation Rate and Clot Retraction Time, Am. J. M. Sc. **211**: 325-331, 1946.

LABORATORY METHODS

A SIMPLIFIED TECHNIQUE FOR THE QUANTITATIVE COLORIMETRIC ESTIMATION OF PREGNANDIOL IN URINE

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PREVIOUSLY this laboratory reported a simple method for the qualitative determination of urinary pregnandiol by its reaction with concentrated sulfuric acid.[1, 2] The present report deals with the study of the pregnandiol color reaction which permits its simple quantitative measurement in a colorimeter.

REAGENTS

Toluene, chemically pure

Concentrated hydrochloric acid, chemically pure

0.1 N sodium hydroxide

Two per cent sodium hydroxide in absolute methanol. A 4 to 8 per cent solution of sodium hydroxide in absolute methanol is first prepared by adding sodium hydroxide pellets to an Erlenmeyer flask containing absolute methanol. The mixture is filtered through a dry fritted glass filter to remove the precipitated carbonate. The sodium hydroxide concentration of the filtrate is determined by titration with 0.1 N sulfuric acid. The filtrate is then adjusted to a concentration of 2 per cent with absolute methanol. The solution is freshly prepared every week.

Acetone, chemically pure

Absolute ethyl alcohol

Concentrated sulfuric acid, chemically pure

TECHNIQUE*

A. Hydrolysis and Excretion of Pregnandiol.—

1. One hundred milliliters urine, 50 ml. toluene, 10 ml. concentrated hydrochloric acid, and two glass beads are added to a 500 ml. flat-bottomed Florence flask.

Department of Metabolism and Endocrinology, Research Institute, Michael Reese Hospital. The department is in part supported by the Michael Reese Research Foundation.

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*Based upon the method for pregnandiol extraction by Astwood and Jones[3] and the color reaction of Talbot and co-workers.[4]

2. The flask is connected via a one-holed cork stopper to a Liebig condenser (water-cooled, 400 to 500 mm. jacket length) in vertical position, and the mixture is boiled vigorously over an electric hot plate for fifteen minutes.

3. The flask and its contents are brought to room temperature by cooling under the water tap.

4. The mixture is transferred to a 500 ml. separatory funnel, and the lower layer (urine) is drawn off.

5. The toluene layer and emulsion are washed twice with 15 ml. portions of 0.1 N sodium hydroxide and then twice with 15 ml. portions of distilled water.

B. Precipitation of Impurities.—

1. The washed toluene and emulsion (A, 5) are transferred to a 125 ml. Erlenmeyer flask with two glass beads.

2. The mixture is boiled over an electric hot plate (in the hood).

3. When the water has evaporated and the toluene mixture is boiling smoothly, 10 ml. of 2 per cent sodium hydroxide in absolute methanol are added.

4. The mixture is evaporated until a granular precipitate appears and approximately one-half of the original toluene volume is reached.

5. The toluene mixture is then filtered, while hot, through a fritted glass filter (medium porosity, Pyrex) with mild suction. (If the filtrate has an orange, pink, or brown tinge, steps B, 3, B, 4, and B, 5 must be repeated until the filtrate is yellow or yellow green.)

6. The precipitate (B, 5) is washed with 15 ml. hot toluene.

7. The combined filtrates (B, 5 and B, 6) are then evaporated to dryness over the hot plate (in the hood), a gentle air stream being used to drive off the last traces of toluene. This avoids charring of the residue.

C. Precipitation of Pregnandiol.—

1. Five milliliters acetone are added to the residue (B, 7) and the mixture is warmed over a hot plate until solution is complete.

2. Twenty milliliters 0.1 N sodium hydroxide are added slowly and the mixture is boiled for three minutes on the hot plate.

3. The flask is then placed in a refrigerator (5° C.) for one hour.

D. Isolation of Pregnandiol.—

1. The mixture (C, 3) is filtered through a fritted glass filter (medium porosity, Pyrex) with mild suction.

2. The precipitate (D, 1) is washed with 15 ml. distilled water.

3. The receiving flask is changed, and 10 ml. hot absolute alcohol are passed through the fritted glass filter to dissolve the precipitate.

4. The alcohol filtrate (D, 3) is evaporated to dryness from the receiving flask over an electric hot plate (in the hood).

E. Color Development and Quantitative Measurement.—

1. Ten milliliters concentrated sulfuric acid are added to the residue (D, 4).

2. Color is allowed to develop for one hour.

3. An aliquot of the solution E, 2 is diluted up to a final volume of 5 ml. with concentrated sulfuric acid in a dry absorption tube (Ceneo No. 12344M tubular cell) and the solution is thoroughly mixed.

4. The color is read on a spectrophotelometer* at 430 mμ (concentrated sulfuric acid is used as the blank).

5. The amount of pregnandiol represented by the absorption reading is obtained from the standardization curve. Calculations for the dilutions involved are made to ascertain the pregnandiol present in the original sample.

EXPERIMENTAL

The evidence that pregnandiol is the steroid extracted by this procedure is based on the spectrophotometric absorption and the melting point data which follow.

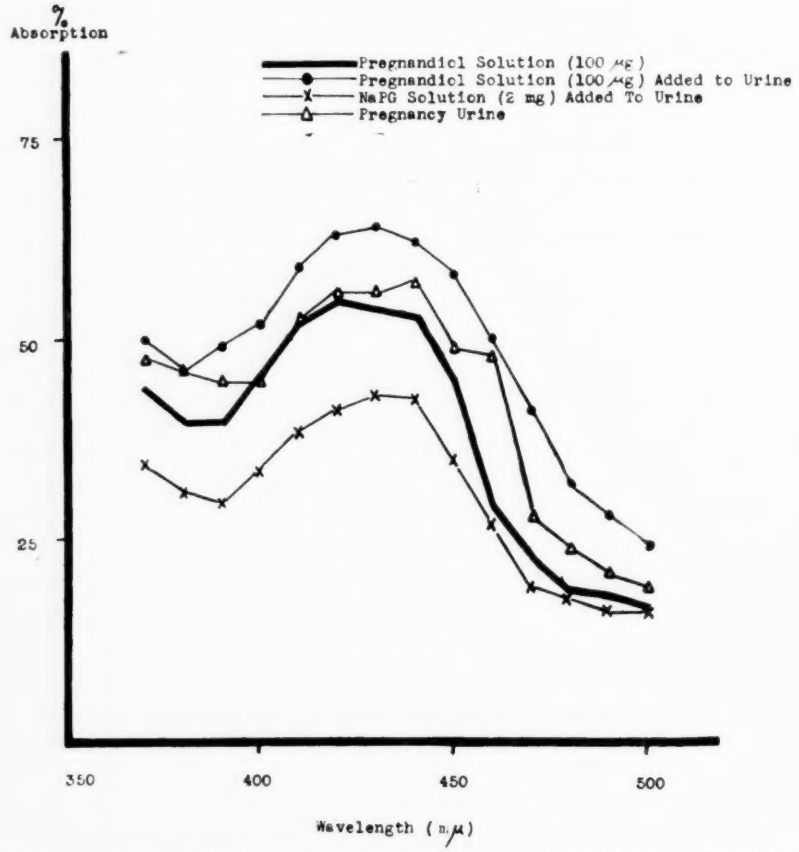

Fig. 1.—Absorption curves of color complexes obtained from the addition of concentrated sulfuric acid to crystalline pregnandiol and pregnandiol recovered from urine.

Pregnancy urine, pooled female urine to which sodium pregnandiol glucuronidate (NaPG)† was added, and pooled female urine to which pregnandiol was added were subjected to the foregoing procedure. The absorption spectra of the color complexes obtained with these specimens and the absorption spec-

*The Cenco-Sheard Spectrophotelometer, Central Scientific Company, Chicago, Ill., was used in the present study.

†Sodium pregnandiol glucuronidate will be designated as NaPG in the succeeding paragraphs.

trum of crystalline pregnandiol to which concentrated sulfuric acid had been added were compared. Fig. 1 illustrates the four curves. They resemble one another closely and demonstrate an absorption maximum between 420 and 440 millimicrons.

The melting points of the products recovered in this experiment are listed below:

1. Pregnancy urine, 205 to 215° C.
2. NaPG added to pooled female urine, 215 to 227° C.

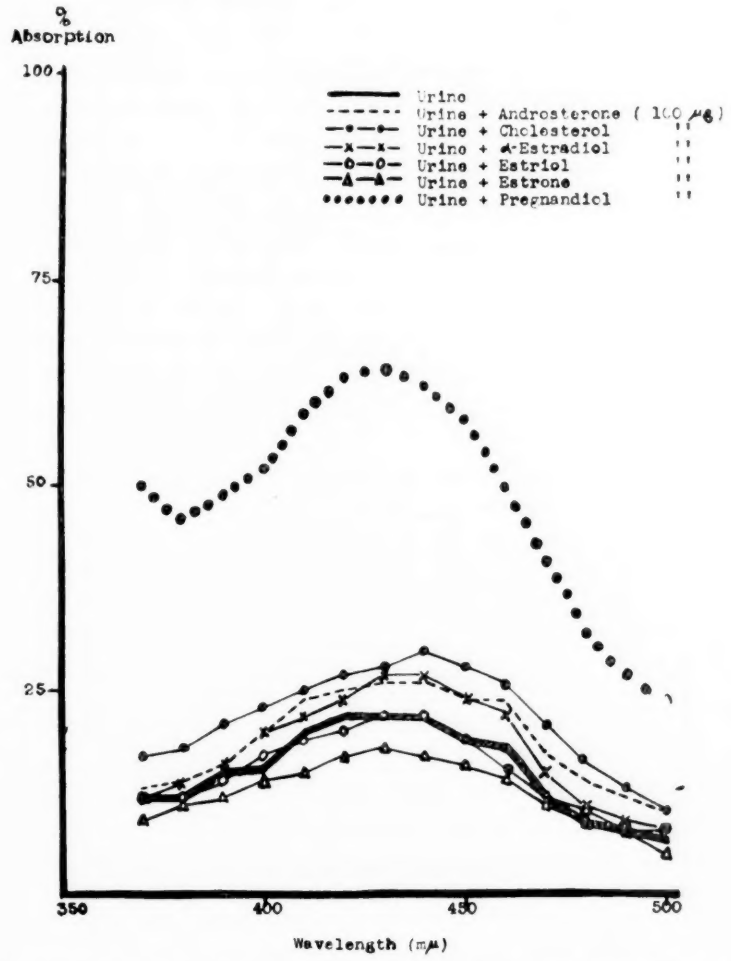

FIG. 2.—Absorption curves of the products recovered from urine to which various steroids had been added.

3. Pregnandiol added to pooled female urine, 227 to 233° C.
4. Crystalline pregnandiol, 234.5 to 237° C.

When the recovered substances (from 1, 2, and 3) were each mixed with pure pregnandiol there was no depression of the melting point.

The absorption curves of other steroids commonly found in urine were studied in similar fashion. Androsterone, cholesterol, α-estradiol, estriol, estrone, and pregnandiol,* 100 μg of each in toluene solution, were added to 100 c.c. samples of pooled female urine and the mixture was run through the recommended procedure. Fig. 2 illustrates the absorption curves obtained. It appears from these data that the steroids tested do not significantly alter the absorption curve (at the given wave lengths) of the pooled female urine used as a control. Only the specimen with added pregnandiol demonstrated a marked increase in absorption with a maximum at 430 millimicrons.

These data appeared to indicate that the method outlined leads to the recovery of pregnandiol alone.

Optimal conditions for the quantitative extraction of pregnandiol from urine and the maximal final color development were established by the following experiments.

I. Duration of Simultaneous Hydrolysis and Extraction.—NaPG (aqueous solution), 2 mg., was added to 100 ml. portions of pooled female urine.† The specimens were refluxed for five, fifteen, and thirty minutes each and then carried through the procedure. The results obtained indicated that maximal extraction of pregnandiol occurs if the urine mixture is refluxed for fifteen minutes. Five minutes of boiling appeared inadequate for optimal recovery of pregnandiol; refluxing for thirty minutes did not interfere with the maximal recovery. It was therefore concluded that specimens should be refluxed for at least fifteen minutes.

II. Efficiency of One Period of Hydrolysis and Extraction.—Toluene, 50 ml., was added to the urine layers of the specimens (I) which had been hydrolyzed and extracted for fifteen minutes. Some of the samples were extracted in the cold for fifteen minutes on a mechanical shaker. The remainder were refluxed for an additional fifteen minutes as in the original procedure. The toluene layers were saved and carried through the technique. The absence of color development at step E, 2 indicated that in the first period of boiling the extraction of urinary pregnandiol was complete.

III. The Effect of Aqueous Sodium Hydroxide.—

A. A series of specimens were carried to step A, 5. At that point the toluene layers were each washed with one, two, and four 15 ml. portions of 0.1 N sodium hydroxide. The technique was then followed as outlined. The results of this experiment showed that at least two washings of the toluene layer with 0.1 N sodium hydroxide are necessary for uniform neutralization. Additional washings with 0.1 N sodium hydroxide did not affect pregnandiol recovery.

B. The combined sodium hydroxide and water washings of the specimens (III A) were diluted to a volume of 100 ml. with distilled water. Ten mil-

*The androsterone, α-estradiol, estriol, and estrone were supplied through the courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J. The pregnandiol and sodium pregnandiol glucuronidate were generously supplied by Ayerst, McKenna and Harrison, Ltd. Montreal, Canada.

†Unless otherwise indicated, in the following experiments 2 mg. sodium pregnandiol glucuronidate were added to 100 ml. portions of pooled female urine. All tests were carried out in duplicate.

liters hydrochloric acid (concentrated) and 50 ml. of toluene were added. These mixtures were carried through the procedure (starting at A, 1). No color development occurred in any specimen. Thus, it appeared that the sodium hydroxide originally used did not remove pregnandiol from the toluene layer.

IV. Precipitation of Impurities by 2 Per Cent Sodium Hydroxide in Absolute Methanol.—

A. One hundred milliliter portions of pooled female urine to which no NaPG was added were carried to step B, 3. At that point, 0, 5, 10, 20, and 30 ml. aliquots of 2 per cent sodium hydroxide in absolute methanol were added and the procedure was completed. When the methanolic sodium hydroxide solution was not employed, the red and violet urinary pigments extracted by the toluene were not removed from solution and they interfered with the final color reaction by producing bizarre colors. The use of sodium hydroxide in methyl alcohol removed these urinary pigments and resulted in constant final color development. Therefore, it was concluded that methanolic sodium hydroxide should be employed at this stage.

B. When the same experiment was carried out with urine specimens to which NaPG had been added, the following observations were made. When 5 ml. of sodium hydroxide in methanol were used, the color reaction developed represented more than 100 per cent recovery of pregnandiol.

Since the control urines (IV A) indicated that methanolic sodium hydroxide is necessary to remove interfering substances, it appeared that 5 ml. of sodium hydroxide in methanol might have been inadequate to remove all the nonpregnandiol chromogens. The addition of 10 ml. of sodium hydroxide in methanol resulted in pregnandiol recoveries of over 90 per cent. The use of 20 to 30 ml. of sodium hydroxide in methanol at step B, 3 reduced the pregnandiol recovery. It was possible to recover pregnandiol from the sodium hydroxide precipitates (at step B, 5) of the specimens in which 20 and 30 ml. of methanolic sodium hydroxide had been used. It was concluded, therefore, that 10 ml. of 2 per cent sodium hydroxide in absolute methanol would be adequate to remove interfering substances without precipitating pregnandiol prematurely.

V. Period of Precipitation.—A series of specimens was carried to step C, 1. In the original communication[1] it was suggested that aqueous 0.1 N sodium hydroxide be added to the warmed acetone solution to precipitate the pregnandiol. Our quantitative studies indicated that under such conditions the precipitation of pregnandiol reached a maximum after three hours of refrigeration at 5° C. However, it was found that if the sodium hydroxide-acetone mixture were boiled three minutes, the maximal pregnandiol precipitation occurred in one hour. Extension of the boiling period led to the precipitation of nonpregnandiol chromogens which interfered with the final color reaction.

VI. Color Development With Sulfuric Acid.—The color reactions developed by solutions containing various amounts of crystalline pregnandiol (8, 40, and 80 γ) were examined on the colorimeter at frequent intervals starting ten minutes after the addition of the acid. Maximal absorption at 430 mμ occurred

between thirty and sixty minutes later. Since the more concentrated specimens reached their maximal absorption with one hour of development, we employed this standard time interval for reading all our specimens on the spectrophotelometer.

VII. Standardization Curves.—Aliquots of a solution of pregnandiol in absolute alcohol (100 μg per cubic centimeter) were measured from a microburette into test tubes. The alcohol was evaporated from the tubes in a glycerine bath. After cooling, 5 ml. of sulfuric acid (concentrated) were buretted into each tube. The color was allowed to develop for one hour (the tubes being shaken occasionally) and was read on the spectrophotelometer at 430 mμ, with concentrated sulfuric acid used as a blank. Fig. 3 represents the average results obtained. In the range of 10 to 100 μg per unit volume (5 ml.), the concentration is proportional to the log of the per cent absorption, obeying Beer's law. With concentrations greater than 100 μg this relationship does not obtain. A similar standardization curve was obtained when pregnandiol was recovered from pooled female urine to which NaPG had been added in varying amounts.

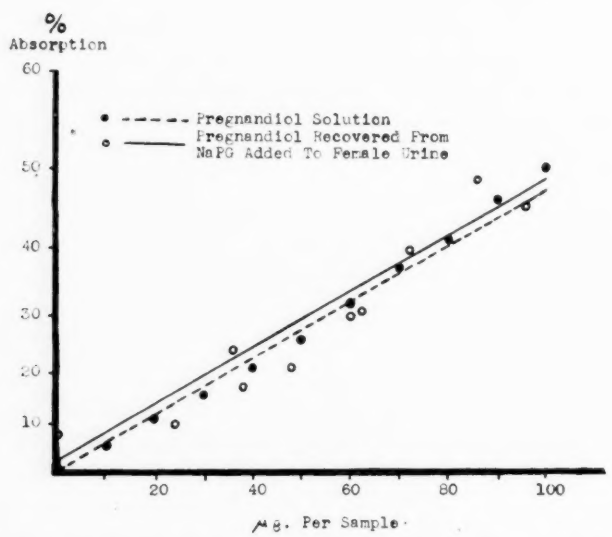

Fig. 3.—Standardization curves of the color reaction (with sulfuric acid) of crystalline pregnandiol and pregnandiol recovered from female urine to which NaPG had been added.

The curve is also illustrated in Fig. 3. Each point represents the average values of five to ten results. This curve parallels that of pure pregnandiol and indicates that the amount of pregnandiol recovered varies directly with the NaPG present in urine.

To determine the efficiency of the method in recovering pregnandiol from NaPG added to urine, the following calculations were considered.

1. Sodium pregnanolone glucuronidate constitutes 20 per cent of NaPG.[6]

2. The molecular weight of pregnandiol is 320. The molecular weight of pure sodium pregnandiol glucuronidate is 536.[7]

Therefore the pregnandiol theoretically obtainable from sodium pregnandiol glucuronidate can be calculated from the following equation (where A = weight of NaPG [in mg.]):

$$\frac{\text{M.W. (pregnandiol)}}{\text{M.W. (NaPG)}} \times (A - 0.2A) = \text{weight of pregnandiol (in mg.)}.$$

Table I represents recoveries of pregnandiol from NaPG calculated as indicated. It will be seen that an average of 95 per cent of the theoretic amount available was recovered.

TABLE I. RECOVERY OF PREGNANDIOL FROM SODIUM PREGNANDIOL GLUCURONIDATE

NAPG ADDED (MG.)	CALCULATED EQUIVALENT OF PREGNANDIOL (MG.)	PREGNANDIOL RECOVERY	
		(MG.)	(%)
0.50	0.24	0.17	71
0.75	0.36	0.42	117
0.80	0.38	0.30	79
1.00	0.48	0.37	77
1.25	0.60	0.55	92
1.30	0.62	0.57	92
1.50	0.72	0.78	108
1.80	0.86	1.04	120
2.00	0.96	0.95	99
		Average	95

DISCUSSION

Methods for the quantitative determination of urinary pregnandiol, either as the conjugated form, sodium pregnandiol glucuronidate, or as free pregnandiol, have been reported previously.[3, 4, 7-10] The majority of the methods require large volumes of urine, consume several days in performance, and usually depend on a gravimetric technique for measuring the final product. The technique reported at this time requires small urine volumes and consists of technically simple manipulations. One person can perform twelve determinations in a working day. The equipment is standard for chemical laboratories and no special apparatus or chemicals are required. The quantitative estimation is a colorimetric procedure which can be adapted to simple laboratory colorimeters. Although the present report considers results obtained with the Cenco-Sheard spectrophotelometer, equally satisfactory results have been obtained in this laboratory with the simpler Klett-Summerson photoelectric colorimeter (using Filter 42).

In carrying out this procedure, technical difficulties may be prevented if precautions discussed elsewhere are observed.[2] These simple measures consist of (1) the use of chemically pure reagents; (2) the preparation of fresh 2 per cent sodium hydroxide in absolute methanol every week; (3) the use of air stream in preventing charring of residues at steps B, 7 and D, 4; and (4) the use of fritted glass filters.

Emulsions have been encountered infrequently at step A, 5. Urine specimens containing large amounts of albumin and/or blood contribute to such emulsion formation. Filtering the visibly bloody specimens reduces the inci-

dence of emulsions. The addition of 1 to 2 drops of a detergent to the toluene mixture usually breaks up the emulsion and facilitates the procedure.

If it is necessary to interrupt the procedure before completion, this may be done conveniently at steps B, 2, B, 7, and D, 4.

The observations reported in this paper lead us to conclude that the method proposed permits the determination of pregnandiol alone. Androsterone (17-ketosteroids) was not found to interfere with the color reaction. The quantitative estimation of pregnandiol in a given urine specimen depends on its spectrophotometric absorption (at 430 mμ). This reading is compared with the standard curve which is a representation of the actual amount of pregnandiol recoverable from urine to which sodium pregnandiol glucuronidate has been added.

Failure to use pregnandiol as a standard or to read the color absorption at 430 mμ has led to results which appear to us to have been interpreted erroneously.[11, 12] Reinhart and Barnes[12] utilized 2 per cent potassium dichromate in water (at 420 mμ) as a standard for measuring pregnandiol. Since this solution is fifty times more concentrated than the amount of potassium dichromate equivalent to the pregnandiol standard previously recommended by this laboratory (0.4 mg. pregnandiol)[2] it is not surprising that their results were

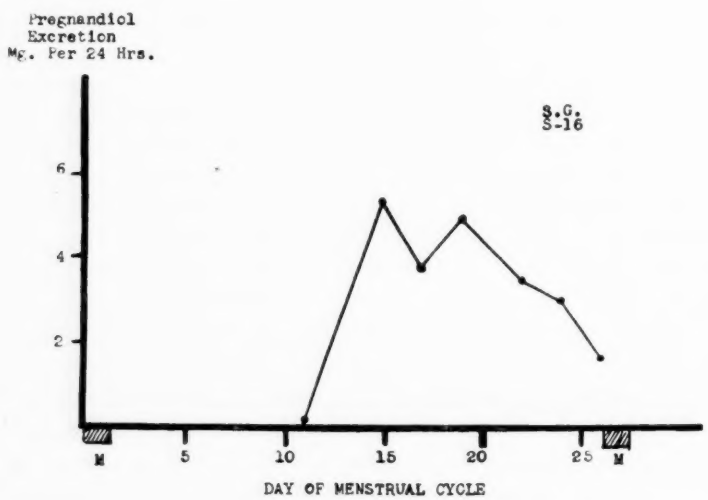

Fig. 4.—Pregnandiol excretion in normal menstrual cycle (present method).

not consonant with those reported from this laboratory. Others have not only employed potassium dichromate as a standard but have read the colors at higher wave lengths (530 to 570 mμ).[11] Since pregnandiol absorption in this region is minimal, we feel that the authors were probably measuring substances other than pregnandiol.

The results of quantitative estimations in normal pregnancy in our laboratory are consistent with those obtained by others (Table II). The pattern and levels of pregnandiol excretion in the normal menstrual cycle coincide with

those reported by Venning and Browne[14] (see Fig. 4). These observations, which will be published elsewhere in extenso, further strengthen our conclusion that the method proposed is accurate, specific, and practical for research and routine clinical use.

TABLE II. COMPARISON OF PREGNANDIOL EXCRETION VALUES IN NORMAL PREGNANCY*

DAYS†	PREGNANDIOL (MG. PER 24 HR.)	
28-56	5-11‡	9-16§
56-84	6-20	15-38
84-112	10-28	19-36
112-140	13-35	27-46
140-168	22-52	21-62
168-196	44-72	30-72
196-224	48-85	41-86
224-252	55-95	56-108
252-280	60-105	50-105

*The two sets of data given in this table are at first glance not strictly comparable. The Venning procedure calculates the pregnandiol concentration from NaPG which contains about 20 per cent of sodium pregnanolone glucuronidate.[5, 6] The method here reported estimates pregnandiol only. It would therefore be expected that the pregnandiol values by the Venning method should be about 20 per cent higher than with the present method. The reason for the virtual coincidence of the values remains at present unexplained.

†Counted from the first day of the last normal menstrual period.

‡From Browne, Henry, and Venning.[13] Venning method (eight cases).

§This laboratory. Present method (four cases).

SUMMARY

A method for the simple quantitative colorimetric determination of urinary pregnandiol is reported.

Spectrophotelometric and melting point data are presented as evidence that pregnandiol alone is the substance measured.

Optimal conditions for the quantitative extraction of pregnandiol and color development are established.

Data presented indicate that an average of 95 per cent of the theoretic pregnandiol available is recovered by the procedure reported.

The results of pregnandiol determinations in the menstrual cycle and in normal pregnancy check closely with observations made in other laboratories which employed the longer gravimetric procedure.

We wish to thank Dr. Rachmiel Levine for his advice and encouragement in this work.

REFERENCES

1. Guterman, H. S.: A Human Pregnancy Test Based Upon a Color Reaction of Pregnandiol in Urine, J. Clin. Endocrinol. **4**: 262-267, 1944.
2. Guterman, H. S.: Further Observations on the Value of the Pregnandiol Color Test for Pregnancy, J. Clin. Endocrinol. **5**: 407-411, 1945.
3. Astwood, E. B., and Jones, G. E. S.: A Simple Method for the Quantitative Determination of Pregnandiol in Human Urine, J. Biol. Chem. **137**: 397-407, 1941.
4. Talbot, N. B., Berman, R. A., MacLachlan, E. A., and Wolfe, J. K.: The Colorimetric Determination of Neutral Steroids (Hormones) in a 24-Hour Sample of Human Urine (Pregnanediol; Total, Alpha and Beta Alcoholic, and Non-Alcoholic 17-Ketosteroids), J. Clin. Endocrinol. **1**: 668-673, 1941.
5. Tarrian, G. F., and Gough, N.: The Isolation of Pregnane-3(α),-o1-20-one from the Hydrolysis Products of "Sodium Pregnanediol Glucuronidate," Biochem. J. **40**: 376-380, 1946.

6. Sutherland, E. S., and Marrian, G. F.: The Separation of Non-Ketonic and Ketonic Sodium Glucuronidates From the "Sodium Pregnanediol Glucuronidate" of Human Pregnancy Urine. The Isolation of Pure Sodium Pregnane-3(α), 20(α)-diol Glucuronidate, Biochem. J. **41**: 193-196, 1947.
7. Venning, E. H.: Gravimetric Method for the Determination of Sodium Pregnandiol Glucuronidate (An Excretion Product of Progesterone), J. Biol. Chem. **119**: 473-480, 1937.
8. Beall, D.: A Method for the Isolation of Pregnandiol From Human Pregnancy Urine, Biochem. J. **31**: 35-40, 1937.
9. Weil, P. G.: A Method for the Isolation of Pregnandiol From the Urine of Pregnant Mares, Proc. Soc. Exper. Biol. & Med. **38**: 503-504, 1938.
10. Hechter, O.: Comparison Between Gravimetric and Reduction Methods for Determination of Pregnandiol Glucuronidate, Proc. Soc. Exper. Biol. & Med. **49**: 299-302, 1942.
11. Morrow, A. G., and Benua, R. S.: An Evaluation of the Guterman Pregnancy Test, Am. J. Obst. & Gynec. **51**: 685-691, 1946.
12. Reinhart, H. L., and Barnes, A. C.: An Evaluation of the Guterman Pregnancy Test in Clinical Practice, J. Clin. Endocrinol. **6**: 664-667, 1946.
13. Browne, J. S. L., Henry, J. S., and Venning, E. H.: The Significance of Endocrine Assays in Threatened and Habitual Abortion, Am. J. Obst. & Gynec. **38**: 927-955, 1939.
14. Venning, E. H., and Browne, J. S. L. : Studies on Corpus Luteum Function: Urinary Excretion of Sodium Pregnandiol Glucuronidate in Human Menstrual Cycle, Endocrinology **21**: 711-721, 1937.

STUDIES IN SERUM PROTEINS

II. A Rapid Clinical Method for the Accurate Determination of Albumin and Globulin in Serum or Plasma

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ELECTROPHORETIC analysis of serum has demonstrated that "serum albumin," as determined by the Howe sodium sulfate method, actually includes both albumin and alpha globulin.[1-3] Other methods for determining albumin values which approximate those obtained by electrophoresis have certain disadvantages. Precipitation of globulins by methanol, as devised by Pillemer and Hutchinson,[4] is difficult technically since it involves working between 0 and 1° C. and, even when these conditions are maintained, occasionally yields erratic results.[1, 3] Popjak and McCarthy's method[5] for separating albumin and globulin with saturated magnesium sulfate involves a delay of twelve hours before filtration can be carried out. In addition, the albumin in the filtrate cannot be estimated by the biuret reaction, but requires a Kjeldahl nitrogen determination. The 28 per cent sodium sulfate method of Milne[6] also requires an overnight delay for precipitation of the globulins. Chow has reported a quantitative immunochemical reaction[7] which, however, employs biologic material of unstable titer and uncertain composition.

The method described below appears to overcome these difficulties. The procedure is based on our observation that sodium sulfite at a concentration of 26.88 per cent precipitates globulin from serum, plasma, or plasma fractions. The earlier sodium sulfite method of Campbell and Hanna was adjusted to give results approximating Howe fractions.[8] Following precipitation of the globulin component, immediate filtration yields a solution in which only albumin is found. The albumin in the filtrate may be determined by the biuret reaction of Weichselbaum[9] or by any other convenient method.

Analyses were performed on human plasma and on subfractions of plasma by the electrophoretic and sodium sulfite methods. The results obtained are compared in Tables I and II.

It will be seen from the results in Table I, obtained on plasma fractions, that albumin is not precipitated by 26.88 per cent sodium sulfite to any extent, whether in pure solution or combined with mixtures of globulins. All globulins were precipitated, regardless of the distribution of globulin subfractions. Although fractions IV-4 and IV-6 contained large amounts of alpha globulin

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TABLE I. COMPARISON OF ELECTROPHORETIC AND SODIUM SULFITE ANALYSIS OF PURIFIED PLASMA FRACTIONS*

FRACTION	ANALYTIC METHOD	ALBUMIN† (GM. %)	TOTAL GLOBULIN (GM. %)	ALPHA GLOBULIN (GM. %)	BETA GLOBULIN (GM. %)	GAMMA GLOBULIN (GM. %)
Albumin‡	Electrophoresis	4.90	0.00	0.00	0.00	0.00
	Sodium sulfite	4.90	0.00			
Gamma globulin§	Electrophoresis	0.00	5.30	0.00	0.00	5.30
	Sodium sulfite	0.00	5.30			
Mixture of albumin and gamma globulin‖	Electrophoresis	2.50	2.50	0.00	0.00	2.50
	Sodium sulfite	2.50	2.50			
IV-4	Electrophoresis	0.93	4.90	2.70	2.20	0.00
	Sodium sulfite	0.90	4.93			
IV-6	Electrophoresis	0.33	3.37	3.04	0.33	0.00
	Sodium sulfite	0.20	3.50			
IV-7	Electrophoresis	0.45	4.05	0.36	3.69	0.00
	Sodium sulfite	0.75	3.75			
IV-8	Electrophoresis	3.87	0.43	0.30	0.13	0.00
	Sodium sulfite	4.05	0.25			
Average	Electrophoresis	1.85	2.94	0.91	0.91	1.11
	Sodium sulfite	1.90	2.89			

*The electrophoretic analyses of purified plasma fractions quoted here are average results obtained for these fractions. The lyophiled fractions supplied were dissolved in 0.15M NaCl before analysis and filtered to remove residual turbidity. A sample of fraction V-1 could not be analyzed because the sodium sulfite filtrate was yellow and interfered with the accuracy of the photoelectric determination.

†Includes fast component.

‡Salt-free human albumin (Cutter Laboratories, Berkeley, Calif.) diluted with 0.15M NaCl.

§Immune human globulin (E. R. Squibb & Sons, New York, N. Y.) diluted with 0.15M NaCl.

‖Sample contained 50 per cent salt-free human albumin and 50 per cent immune human globulin made up to a total protein concentration of 5.0 Gm. per 100 c.c. with 0.15M NaCl.

TABLE II. COMPARISON OF ELECTROPHORETIC AND SODIUM SULFITE ANALYSIS OF HUMAN SERA

DIAGNOSIS	ALBUMIN (GM. %)			GLOBULIN (GM. %)			A/G RATIO		
	ELECTROPHORESIS	SODIUM SULFITE	DIFFERENCE BETWEEN ELECTROPHORESIS AND SODIUM SULFITE	ELECTROPHORESIS	SODIUM SULFITE	DIFFERENCE BETWEEN ELECTROPHORESIS AND SODIUM SULFITE	ELECTROPHORESIS	SODIUM SULFITE	DIFFERENCE BETWEEN ELECTROPHORESIS AND SODIUM SULFITE
Multiple myeloma	2.02	2.00	+0.02	4.84	4.70	+0.14	0.42	0.43	−0.01
Multiple myeloma	1.97	2.30	−0.33	3.06	2.90	+0.16	0.64	0.79	−0.15
Multiple myeloma	1.80	1.50	+0.30	8.25	8.90	−0.65	0.22	0.17	+0.05
Rheumatic fever	1.65	1.75	−0.10	5.05	5.15	−0.10	0.33	0.34	−0.01
Rheumatic fever	3.10	3.10	0.00	5.14	5.40	−0.26	0.60	0.57	+0.03
Rheumatoid arthritis	2.10	2.20	−0.10	5.52	5.60	−0.08	0.38	0.39	−0.01
Hepatic cirrhosis	2.71	2.70	+0.01	5.85	6.00	−0.15	0.46	0.45	+0.01
Pooled normal serum	4.00	3.70	+0.30	3.40	3.25	+0.15	1.18	1.14	+0.04
Average	2.42	2.41		5.14	5.24		0.47	0.46	

which, by the Howe method, is included in the "albumin" fraction, sodium sulfite fractionations gave good agreement with electrophoretic values.

The human sera analyzed (Table II) consisted of six samples of known electrophoretic composition made available to us* and two of our samples which were analyzed electrophoretically elsewhere.† Mean values for albumin, globulin, and A/G ratio did not differ from those obtained by electrophoresis by more than 2.2 per cent.

Only one sample of pooled normal serum (Table II) was analyzed both electrophoretically and chemically. However, a number of analyses have been carried out on pools of serum obtained from fifty to one hundred fifty donors; these analyses gave an average value of 53 per cent albumin, with individual pools varying from 51 to 55 per cent albumin. These values coincide well with the value of 52 per cent albumin obtained for plasma from normal adults by electrophoresis.[10]

PROCEDURE

Reagents.—

Twenty-eight per cent sodium sulfite solution. Dissolve exactly 28.00 Gm. of anhydrous sodium sulfite in distilled water at 28° C. The salt is difficultly soluble but will go into solution with sufficient shaking. Make up to 100 ml. and store at room temperature.

Biuret reagent (Weichselbaum).[9]

Determination.—

1. Place exactly 24 ml. of sodium sulfite solution in a 25 ml. graduated mixing cylinder. Add 1.0 ml. of serum or plasma and mix well by inversion. Do not shake.
2. Filter immediately through a double thickness of folded No. 42 Whatman filter paper. The filtrate should be crystal clear. If the first few drops are turbid, they should be refiltered.
3. Discard the first 5 ml. of filtrate. (A certain proportion of the albumin in the first few milliliters of filtrate will be adsorbed on the filter paper and low albumin values will be obtained if this material is used for analysis.)
4. To 5 ml. of Weichselbaum's biuret reagent in a cuvette add 5 ml. of filtrate. Mix well by shaking.

(The blank is prepared with 5 ml. of biuret reagent and 5 ml. of 28 per cent sodium sulfite.)

5. After standing thirty minutes, the solution is read on a photoelectric colorimeter or spectrophotometer at a wave length of 540 millimicrons.
6. The value obtained in the preceding step is that for serum albumin. Globulin is determined as the difference between total protein and albumin values.

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†With the cooperation of Miss Miriam Reiner of the Biochemistry Laboratory, Mount Sin[ai] Hospital, New York, N. Y.

SUMMARY

A method for the determination of albumin in serum, plasma, or plasma fractions is described. This method gives reproducible results which agree closely with those obtained by electrophoretic analysis. As the procedure is rapid and technically simple, it is suitable for use in the routine clinical laboratory.

We wish to thank Dr. Philip P. Cohen of the Department of Physiological Chemistry, University of Wisconsin, Miss Miriam Reiner of the Biochemistry Laboratory, Mount Sinai Hospital, New York, N. Y.; Dr. A. N. Kutz and Dr. J. D. Perrings of the Biochemical Section, The Armour Laboratories, Chicago, Ill.; and the members of the Samuel Deutsch Serum Center, Michael Reese Research Foundation, for their cooperation in supplying samples for analysis and providing analyzed samples.

The products of plasma fractionation employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

REFERENCES

1. Cohn, C., and Wolfson, W. Q.: Studies in Serum Proteins. I. The Chemical Estimation of Albumin and of the Globulin Fractions in Serum, J. LAB. & CLIN. MED. **32**: 1203, 1947.
2. Luetscher, J. A., Jr.: Biological and Medical Applications of Electrophoresis, Physiol. Rev. **27**: 621, 1947.
3. Petermann, M. L., Young, N. F., Hoagness, K. R., and Rhoads, C. P.: The Determination of Plasma Albumin by Chemical and Electrophoretic Methods, Proc. Am. Federation Clin. Research **3**: 75, 1947.
4. Pillemer, L., and Hutchinson, M. C.: The Determination of the Albumin and Globulin Contents of Human Serum by Methanol Precipitation, J. Biol. Chem. **158**: 299, 1945.
5. Popjak, G., and McCarthy, E. F.: Osmotic Pressures of Experimental and Human Lipaemic Sera. Evaluation of Albumin-Globulin Ratios With the Aid of Electrophoresis, Biochem. J. **40**: 789, 1946.
6. Milne, J.: Serum Protein Fractionation: A Comparison of Sodium Sulfate Precipitation and Electrophoresis, J. Biol. Chem. **169**: 595, 1947.
7. Chow, B. F.: The Determination of Plasma or Serum Albumin by Means of Precipitin Reaction, J. Biol. Chem. **167**: 757, 1947.
8. Campbell, Walter R., and Hanna, Marion I.: The Albumin, Globulins, and Fibrinogen of Serum and Plasma, J. Biol. Chem. **119**: 15, 1937.
9. Weichselbaum, T. E.: An Accurate and Rapid Method for the Determination of Proteins in Small Amounts of Blood Serum and Plasma, Am. J. Clin. Path. (Tech. Supp.) **10**: 40, 1946.
10. Brand, E., and Edsall, J. T.: The Chemistry of the Proteins and Amino Acids, Ann. Rev. Biochem. **XVI**: 223, 1947.

SYSTEMATIC QUALITATIVE ANALYSIS OF BIOLOGIC MATERIALS FOR COMMON STEAM-VOLATILE ORGANIC POISONS

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TOXICOLOGIC analysis of biologic material is sometimes required when no indication or circumstantial evidence of the presence or absence of a specific poison is available. While adequate systematic qualitative analytic procedures for inorganic poisons such as heavy metals are available, the search for organic poisons necessitates a laborious, time-consuming, step-by-step analysis for each individual compound.

Organic poisons may be divided into the following groups: (1) steam-volatile compounds; and (2) nonvolatile compounds extractable with suitable organic solvents from (a) aqueous acid solution and (b) aqueous alkaline solution.

An attempt has been made to develop methods for the systematic detection of various compounds found in each of these groups. In the present paper a simple scheme for the detection of the more common steam-volatile poisons is described.

The following compounds are included in the scheme of analysis:

Acid steam distillate: phenol, cresol (tricresol), thymol, aniline, hydrocyanic acid, chloroform, chloral hydrate, carbon tetrachloride, acetaldehyde, ethyl alcohol, formaldehyde, methylalcohol, nitrobenzene, carbon disulfide.

Alkaline steam distillate: nicotine, amphetamine, aniline.

EXPERIMENTAL

The acid steam distillate is obtained in the following manner. The tissue to be examined (100 Gm.) is homogenized in a Waring blendor with distilled water (200 ml.) and the mixture is adjusted with tartaric acid to a pH of 5.0. The homogenate is transferred to a distilling flask (1 liter) which is part of an all-glass steam distilling apparatus.* About 5 Hengar boiling granules are added to the steam-generating flask. All joints are lubricated with a thin layer of silicone. The distillate is collected in a suitable cooled container with the end of the condenser immersed in distilled water (10 ml.). The steam distillation is carried out until 200 ml. have been collected.

The homogenate is then made alkaline with magnesium oxide to a pH of 8.0. The steam distillation is repeated and 200 ml. of distillate again are collected.

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*The apparatus, designed for this procedure according to our specifications, was obtained from E. Machlett & Sons, New York, N. Y.

Quantities of tissue weighing less than 100 Gm. may be analyzed by employing proportionately smaller volumes of water in preparing the homogenate and by collecting appropriately reduced volumes of distillate. However, a minimum of 25 Gm. of tissue is recommended for a complete analysis.

In the literature of this subject are described a large number of tests for the detection of the poisons considered in this scheme. However, many of these tests proved to be unsatisfactory, either because of insufficient sensitivity or because they gave positive or equivocal results with steam distillates from tissues containing no poison. Since each of those tests which was found suitable has been modified, a detailed description of each procedure is given. Reference is made to the papers in which the tests were originally described. The lowest concentration of the compound at which the test is definitely positive is listed. This concentration is considered the practical limit of sensitivity. None of the tests described give positive results with steam distillates from homogenates of liver containing no poison.

1. *Precipitation Test With Bromine Water.*[1]—To the distillate (1 ml.) add bromine water (1 drop). If no precipitation occurs, add additional reagent (2 or more drops). A positive reaction is indicated by the formation of a white or yellowish precipitate or by a definite cloudiness of the solution.

Sensitivity: Phenol, 0.0025 per cent; tricresol, 0.0020 per cent; thymol, 0.0015 per cent; aniline, 0.0015 per cent.

2. *Millon's Test.*[2]—To the unknown solution (3 ml.) in a small test tube add the reagent (3 drops). Heat the mixture, at first gently, then with a gradually increasing intensity, while observing carefully for changes in color. A positive reaction is indicated by a red or red-brown color or a precipitate. A bright yellow precipitate may form; this may consist of mercury salts and does not indicate a positive test.

Sensitivity: Phenol, 0.0007 per cent; tricresol, 0.0007 per cent; aniline, 0.0050 per cent.

3. *Prussian Blue Test for Cyanide.*[3, 4]—To the unknown (3 ml.) in a test tube add a solution of potassium hydroxide (5 per cent, 3 or 4 drops), then a freshly prepared solution of ferrous sulfate (2 per cent, 1 or 2 drops), and a solution of ferric chloride (1 per cent, 1 drop). Shake the solution and warm gently. Acidify carefully with hydrochloric acid (2N). If much hydrocyanic acid is present, a precipitate of prussian blue (ferric ferrocyanide) will appear at once, but if the concentration is weak the colloidal solution will have merely a blue, blue-green, or green-blue color. After some time a flocculent precipitate of prussian blue will settle to the bottom of the tube. Prussian blue will not appear if the reaction is alkaline. On the other hand, in strongly acid solutions the formation of prussian blue is delayed. Therefore, the final mixture should be adjusted (with indicator paper) to the optimal range of pH 2 to pH 5.

The limiting concentration of hydrocyanic acid in this test has been stated to be 1:50,000 (0.002 per cent)[3]; for practical purposes, however, the sensitivity is limited to a concentration of 0.004 per cent.

4. *Alkaline Pyridine Test.*[5]—To the unknown solution (2 ml.) in a medium-sized test tube add pyridine (0.4 ml., reagent grade) and mix thoroughly. Add a solution of potassium hydroxide (40 per cent, 4 ml.) and heat one to three minutes in a boiling water bath while observing the pyridine layer for color changes. A red color developing in the pyridine layer within three minutes signifies a positive test. The color may fade with continued heating and it is essential, therefore, to observe the reaction carefully.

Sensitivity: Chloroform, 0.0002 per cent; chloral hydrate, 0.0001 per cent; carbon tetrachloride, 0.025 per cent.

5. *Direct p-Hydroxydiphenyl Test.*[6, 18b]—To the distillate (1 drop) add a solution of copper sulfate (1 per cent, 1 drop) and then concentrated sulfuric acid (1 ml.). Immediately cool the tube with ice water and add a drop of the p-hydroxydiphenyl reagent (p-hydroxydiphenyl, 1.5 per cent, in a solution of sodium hydroxide, 0.5 per cent). Shake the mixture thoroughly and allow it to stand for twenty minutes.

A blue color is formed in the presence of acetaldehyde; this changes gradually to bright violet (maximal color intensity occurs after twenty to thirty minutes); with formaldehyde a bright green color is produced, but this changes to blue on standing.

Sensitivity: Formaldehyde, 0.0002 per cent; acetaldehyde, 0.0008 per cent.

6. *p-Hydroxydiphenyl Test Following Oxidation.*[6]—In a large test tube place the distillate (0.1 ml.), a solution of potassium permanganate (5 per cent, 1 drop), and phosphoric acid (10 per cent, 1 drop). After mixing, the solution is allowed to stand at room temperature for one minute. Add a solution of oxalic acid (saturated) drop by drop, with shaking, until the solution is decolorized. After decolorization has occurred, add concentrated sulfuric acid (6 drops) while agitating the tube in ice water. Remove the tube from the bath, add a solution of copper sulfate (1 per cent, 1 drop), and mix. Replace the tube in ice water and agitate it while adding concentrated sulfuric acid (3 ml.). Remove the tube from the bath, add a drop of the p-hydroxydiphenyl solution (as in Test 5), mix well, and allow to stand at room temperature. Record any production of color after twenty to twenty-five minutes.

In the presence of methyl alcohol a bright green-blue to blue color is produced, while with ethyl alcohol a bright blue-violet color appears.

Sensitivity: Methanol, 0.0008 per cent; ethanol, 0.004 per cent.

7. *Indophenol Test.*[7]—To the unknown solution (3 ml.) slowly add concentrated H_2SO_4 (3 ml.) while cooling the tube in a beaker of water. Then add a small amount of sodium nitrite and mix. Allow to stand at room temperature for five minutes and observe the color.

In the presence of phenol in a concentration of 0.01 per cent or higher a red color is produced. In a still higher concentration (1:1000) other phenols also produce colors: cresol, yellow-brown; thymol, faint yellow.

8. *Ware's*[8] *Test for Phenol.*—To a portion of the distillate (1 ml.) add concentrated HCl (3 ml.). Mix and warm in a water bath at 70° C. for one minute. Quickly add a small amount of Ware's mixture (sodium nitrite, 1 part; sodium nitrate, 1 part; anhydrous sodium sulfate, 2 parts) and let stand at room temperature for five minutes. Observe the color. Cool, make alkaline with a concentrated solution of NH_4OH, and again observe the color.

In the presence of phenol a cherry red color is produced in acid solution and a green color in alkaline solution. The test is sensitive to at least 1 ml. of 0.02 per cent phenol. Tricresol and aniline produce yellow or brown colors, but these are readily distinguished from the colors observed in the presence of phenol.

9. *Selenous Acid Test.*[9]—To a portion of freshly prepared Mecke's reagent (2.5 ml.) add the distillate (0.5 ml.). Mix and observe the color at once. (Mecke's reagent is prepared by dissolving 0.5 Gm. of selenous acid in 100 ml. of concentrated sulfuric acid.)

In the presence of tricresol a distinct red color is produced, whereas phenol is at first yellow-green and gradually becomes red-brown.

Sensitivity: Tricresol, 0.005 per cent.

10. *Hypochlorite test.*[10]—To the distillate (5 drops), in a depression of a white spot plate, add chemically pure aqueous sodium hypochlorite solution (2 drops) and observe any change in color.

If aniline is present in concentrations greater than 0.0025 per cent, a clear violet, gradually changing to a dirty violet color, is produced.

11. *Diazotization and Coupling.*[11]—The distillate (1 drop), in a depression of a white spot plate, is acidified with dilute HCl (1 small drop). Add a freshly prepared solution of sodium nitrite (0.2 per cent, 1 drop) and mix. Destroy the excess nitrous acid with a solution of ammonium sulfamate (1 per cent, 1 drop). Then add freshly prepared coupling reagent, N-(1-naphthyl)-ethylenediamine dihydrochloride (0.2 per cent, 1 drop), and observe the development of color during a period of one to two minutes.

Aniline (and all other primary amines) form diazo compounds with nitrous acid; these are converted to highly colored azo compounds with N-(1-naphthyl)-ethylenediamine. With aniline a purple color is produced which becomes gradually more intense within about one minute after addition of the coupling agent. Phenol, cresol, amphetamine, and nicotine are negative. The test is sensitive to 0.00065 per cent aniline.

12. *Test With Wasicky's reagent.*[12]—To the distillate (1 ml.) add quickly, with mixing, an equal volume of the reagent (β-dimethylaminobenzaldehyde, 2 Gm., in concentrated H_2SO_4, 6 ml., to which water, 0.4 ml., is added). Observe the color immediately.

In the presence of thymol an orange to orange-red color is produced, whereas the control (distilled water) tested in the same manner has a pale-yellow color.

The test is positive to 0.001 per cent thymol. The orange color fades quickly.

13. *Fulton's Test for Phenols.*[13]—To the distillate (5 ml.) add NH_4OH (28 per cent, 5 drops), hydrogen peroxide (3 per cent, 1 ml.), and a solution of copper sulfate (0.1 per cent, 3 drops). Mix and let stand for ten minutes. In the presence of thymol a pink-violet color is produced.

This test is not very sensitive. The absolute limit of sensitivity is a thymol concentration of 0.005 per cent. Other phenols also produce colors; for example, a solution of tricresol produces an orange-pink color which changes to yellow.

14. *Thiocyanate Test.*[14]—To the distillate (3 ml.) in a test tube add KOH (2N, 1 drop) and a solution of yellow ammonium sulfide (a solution of 10 per cent ammonium hydroxide saturated with hydrogen sulfide, 2 drops). Heat gently for two minutes over the flame, and add HCl (4N, 0.5 ml.). Allow the solution to cool; add a solution of ferric chloride (1 per cent, 3 to 5 drops). A red or red-brown color indicates the presence of cyanide.

The test is sensitive to 3 ml. of a 1:70,000 solution of cyanide (calculated as HCN).

15. *Picric Acid Test.*[15]—To the distillate (3 ml.) add a solution of picric acid (saturated, 3 drops) and a solution of sodium carbonate (20 per cent, 3 drops). Warm gently over the flame for a few minutes; allow to stand at room temperature for ten minutes. An orange-red color or precipitate indicates a positive test.

The test is sensitive to 3 ml. of 1:250,000 cyanide solution (calculated as HCN).

16. *Resorcinol Test (Strong Alkali).*[16]—To the distillate (1 ml.) add aqueous resorcinol solution (saturated, 2 drops) and a solution of KOH (20 per cent, 1 ml.). Warm at 60° C. in a water bath for two to three minutes and observe the color of the solution. A red color is produced with chloroform, chloral hydrate, and carbon tetrachloride.

Sensitivity: Chloroform, 0.005 per cent; chloral hydrate, 0.002 per cent; carbon tetrachloride, 0.025 per cent.

17. *Resorcinol Test (Weak Alkali).*[16]—To the distillate (1 ml.) add resorcinol solution (saturated, 2 drops) and a solution of sodium carbonate (20 per cent, 1 ml.). Shake and let stand for twenty minutes at room temperature. Dilute with distilled water (2 or 3 volumes) and observe the tube for green fluorescence. Viewing conditions must be optimal or the fluorescence may be missed. View against a black background with light directed toward the tube at an angle of 90 degrees from the line of vision.

A solution of chloral hydrate in a concentration of 0.001 per cent is sufficient to give a positive test, whereas chloroform and carbon tetrachloride are negative.

18. *Phloroglucinol Test.*[17]—To the distillate (1 ml.) add a small amount of granular phloroglucinol (chemically pure) and a solution of sodium carbonate (20 per cent, 1 ml.). Shake and observe the color during a period of one-half hour at room temperature.

A solution containing as little as 0.001 per cent chloral hydrate will produce an orange-red color. The color of the control is pale-violet. Chloroform and carbon tetrachloride do not give this test.

19. *Chromotropic Acid Test*[18a] (*According to Feigl*[18b]).—The distillate (0.05 ml. or 1 drop), in a test tube, is mixed with a solution of sulfuric acid (72 per cent, 3 ml.); a little solid chromotropic acid (1,8-dihydroxy-naphthalene-3,6-disulfonic acid) is added and the tube is heated in a water bath at 60° C. for ten minutes. A bright violet color appears in the presence of formaldehyde. The test will detect formaldehyde in a 0.0007 per cent solution. Acetaldehyde is negative.

20. *Fuchsin Test.*[18b, 19]—To the distillate (1 ml.) add reduced fuchsin reagent (10 drops) and concentrated H_2SO_4 (5 drops). Let stand at room temperature for twenty minutes. The presence of formaldehyde is indicated by a violet color.

Sensitivity: Formaldehyde, 0.001 per cent; acetaldehyde is negative.

The reagent is prepared as follows. Dissolve basic fuchsin (2 Gm.) in hot water (120 ml.) and allow the solution to cool. Add a solution of sodium sulfate (2 Gm.) in distilled water (20 ml.) and follow with concentrated HCl (2 ml.). Dilute with distilled water to 200 ml. and let stand at least one hour. The freshly prepared solution has a yellow color and should be stored in a cold room.

21. *Oxidation Tests for Methanol.*[18b, 19]—To the distillate (5 ml.) add a solution of potassium permanganate (5 per cent, 3 drops) and a solution of phosphoric acid (10 per cent, 4 drops). Let stand at room temperature for two minutes. Add small amounts of sodium bisulfite, with mixing, until the solution is decolorized (avoid excessive quantities).

Divide the solution in two parts:

(a) To the oxidized solution (3 ml.) add reduced fuchsin reagent (15 drops) and concentration H_2SO_4 (6 drops). Mix and let stand for twenty minutes. If methanol is present in the original solution in a concentration of 0.008 per cent or higher a violet color will appear.

(b) To the oxidized solution (1 ml.) add H_2SO_4 (72 per cent, 4 ml.) and a small amount of chromotropic acid. Warm at 60° C. for ten minutes (see Test 19).

Sensitivity: Methanol, 0.0013 per cent.

22. *Electrolytic Reduction of Nitrobenzene and Detection of the Nitroso Compound Produced.*[18b, 20]—To the distillate (1 ml.) in a small (10 ml.) beaker add the freshly prepared reagent (1 per cent solution of sodium pentacyanoamine ferroate, 1 ml.)[21] and a solution of sodium hydroxide (4N, 1 ml.). A current is then allowed to pass through the solution, using a nickel wire as a cathode and a lead wire as an anode. The source of the current can be either a flashlight battery or a 4-volt storage battery. Allow the electrolysis to proceed for one-half hour. If nitro compounds are present in the original solution

a color will appear during the electrolysis, nitrobenzene producing a bright green color, varying in intensity with the concentration. With high concentrations, a dark-violet color may be produced.

Sensitivity: Nitrobenzene, 0.006 per cent.

23. *Reduction-Diazotization Test.*[11]—To the distillate (6 drops) in a test tube add HCl (2N, 3 drops) and a small amount of zinc powder. Agitate and allow to stand for two to three minutes at room temperature. Remove two drops with a dropper and place them in a depression of a white spot plate; then perform the diazotization test (Test 11).

In this test nitrobenzene is reduced to aniline which is detected by diazotization and coupling. The test is sensitive to a solution of 0.006 per cent nitrobenzene.

24. *Formaldehyde-Plumbite Test.*[18b, 22a]—To the distillate (2 ml.) add bromine water, drop by drop, until the light-yellow color is permanent. Let stand for two minutes. In this way hydrogen sulfide which interferes with the test, is removed by oxidation. Remove the excess bromine by adding a small amount of sodium sulfite. Then add formaldehyde (40 per cent, 5 drops) and alkaline plumbite solution (5 drops). (Prepare the solution by dissolving lead acetate, 1 Gm., in water, 100 ml., and adding KOH, 20 per cent, until the precipitate which forms has dissolved.) Let stand for one-half hour. If large amounts of carbon disulfide are present a black or dark-brown precipitate will form, while with smaller amounts the solution is colored yellow-brown.

Sensitivity: Carbon disulfide, 0.004 per cent.

25. *Ammonium Molybdate Test.*[22b]—To the distillate (2 ml.) add alcoholic potassium hydroxide (10 per cent, 0.5 ml.) and heat in a boiling water bath for five minutes. Cool to room temperature, then add ammonium molybdate solution (2 drops) and acidify with concentrated sulfuric acid added drop by drop. In the presence of carbon disulfide a blue or green-blue color develops which, with very small amounts of carbon disulfide, may form only very slowly. Therefore, allow the tube to stand for two hours at room temperature. A greenish-yellow color may develop in the distilled water control.

Sensitivity: Carbon disulfide, 0.004 per cent.

26. *Precipitation With Nessler's Reagent.*[23]—To the distillate from the alkaline solution (5 ml.) add Nessler's reagent (10 drops). If a precipitate does not appear at once, pack the tube in ice or keep in the refrigerator for at least four hours and again examine for a precipitate.

In a concentration of 0.1 per cent, amphetamine produces an amorphous white precipitate. In a concentration of 0.02 per cent, a white cloud appears. Aniline and nicotine are not precipitated even at concentrations higher than 0.1 per cent.

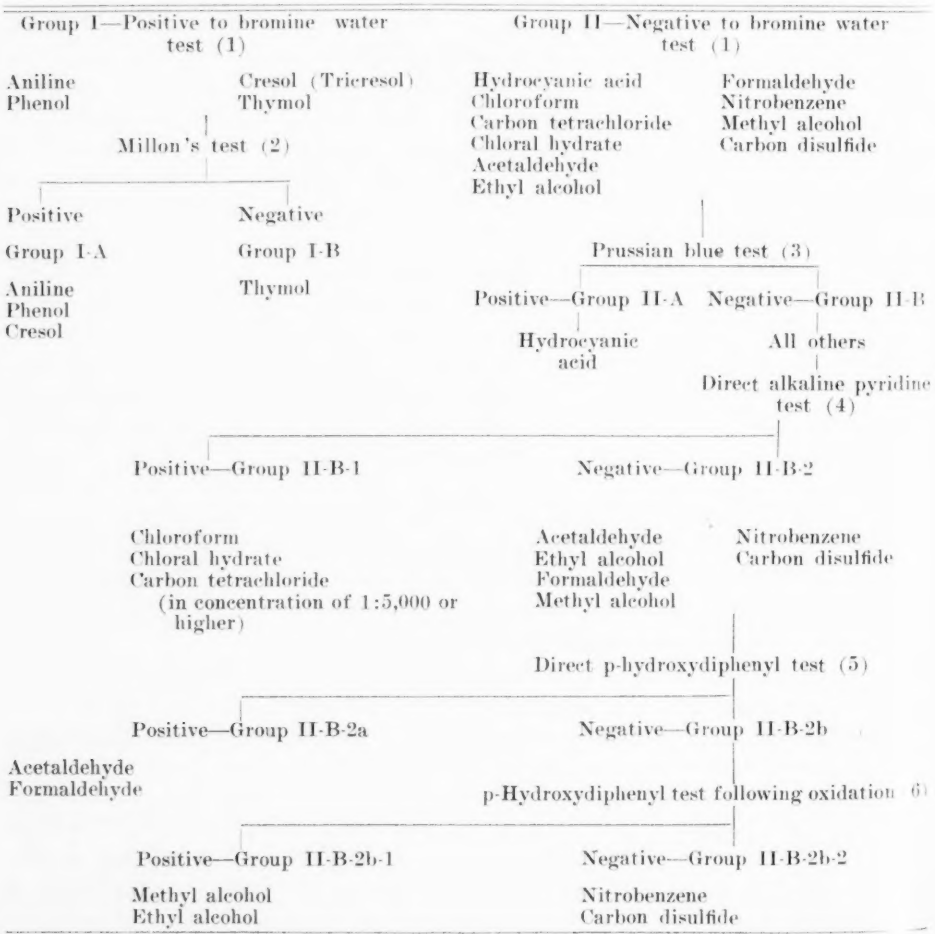
27. *p-Nitroaniline Test.*[24]—The alkaline distillate (15 ml.) is extracted with three portions (5 ml. each) of a mixture of petroleum ether-toluene (1:1) by shaking for five minutes with each extraction. The amine is transferred to

aqueous solution by washing the combined ether-toluene fractions with two portions of HCl (0.2 per cent, 5 ml. each). The acid solution is carefully adjusted to pH 6 to pH 7 with dilute NaOH solution. To the resulting solution (5 ml.) add cold p-nitrobenzenediazonium chloride reagent (5 ml.) and allow to stand for an hour at room temperature. Add a solution of sodium carbonate (1.1 per cent, 5 ml.), let stand for fifteen minutes, and add sodium hydroxide (10 per cent, 1 ml.) to develop the color.

In the presence of amphetamine a distinct red color is produced. Aniline may also produce a red color even when very small amounts are present. Nicotine, however, is negative to this test, or may produce a nondescript greenish-yellow color. A solution of 0.001 per cent amphetamine (sulfate) may be detected.

Stock-Solution: p-Nitroaniline (3.5 Gm.) is suspended in concentrated HCl (5 ml.) by breaking up clumps with a glass rod. The suspension is diluted with distilled water to 500 ml., shaken for twenty minutes, and filtered. The filtrate remains stable when kept in the refrigerator.

TABLE I. GROUP REACTIONS FOR POISONS IN THE ACID STEAM DISTILLATE



Reagent: The stock solution (10 ml.) is cooled (0° C.); concentrated HCl (2 ml.) is added and the mixture is allowed to stand at 0° C. for ten minutes. Freshly prepared sodium nitrite solution (0.7 per cent, 6 ml.) is added, and after another ten minutes at 0° C. the solution is diluted with distilled water to 200 milliliters. The solution is ready for use after it has remained at 0° C. for two hours. When stored in a cold room it remains stable for three weeks.

28. *Picric Acid Precipitation Test.*[25]—To the distillate (5 ml.) add picric acid solution (saturated, 10 drops) and mix. Allow to stand in ice or in the refrigerator for four to six hours. If a precipitate forms, examine it under the microscope.

If nicotine is present characteristic crystals of the picrate will appear. These are long, thin, yellow needles which grow in fan-shaped clusters, resembling sheaves of grain.

Sensitivity: Nicotine, 0.0025 per cent.

Aniline and amphetamine in much higher concentrations may be precipitated by picric acid, but the precipitate is not characteristic in appearance.

29. *Oxidation-Nitroprusside Test.*[26]—To the distillate (5 ml.) add 2 drops of a buffer solution of pH 4.0 (72 ml. of molar acetic acid; 12 ml. of molar sodium hydroxide), potassium persulfate (0.1 to 2 Gm.), and a solution of silver nitrate (1 per cent, 1 drop). Place in a boiling water bath for one minute, shake well, and cool at 20° C. Add crystalline sodium thiosulfate (0.05 to 0.15 Gm.), shake until dissolved, add sodium nitroprusside (10 per cent, 2 to 4 drops), and make slightly alkaline with ammonium hydroxide solution (3 per cent).

In the presence of nicotine a red color appears in two to three minutes. The solution must be slightly acid and must be free from ions which precipitate silver ions. The presence of aniline produces a violet discoloration during the heating, but the color soon changes to yellow. With larger amounts of aniline the solution becomes black and interferes with the test. Amphetamine does not produce a color.

Sensitivity: Nicotine, 0.0025 per cent.

DETECTION OF POISONS IN THE ACID AND ALKALINE STEAM DISTILLATES

In Table I the poisons in the acid steam distillate are classified according to group reactions. (The figures in parentheses refer to the number under which the test is described in the preceding section of this paper.) After a poison has been classified by the group reaction, its presence is ascertained by further differentiating and confirmatory tests (Table II).

DISCUSSION

Phenolic compounds such as salicylic acid, α- and β-naphthol, and pyrogallol are not included in this scheme since, in disagreement with certain textbooks of toxicology,[27] it was found that these compounds are not steam volatile to any significant degree, even if the pH of the solution to be distilled is as low as 2.0.

TABLE II. DIFFERENTIATING TESTS—ACID STEAM DISTILLATE

Group I-A

	INDOPHENOL TEST (7)	WARE'S TEST (8) ACID SOLUTION	WARE'S TEST (8) ALKALINE SOLUTION	SELENOUS ACID TEST (9)	HYPOCHLORITE TEST (10)	DIAZOTIZATION AND COUPLING (11)
Phenol	Red, Red-brown	Cherry-red	Green	Pale-yellow	Neg.	Neg.
Trieresol	Light-brown	Dark-amber	Yellow	Red-amber	Neg.	Neg.
Aniline	Pale-yellow or colorless	Yellow	Bright yellow	Pale-yellow	Violet	Bright purple

Group I-B

Thymol: Test with Wasicky's reagent (12)—orange-red color
Fulton's test for phenols (13)—pink-violet color

Group II-A

Hydrocyanic acid
Thiocyanate test (14)—red or red-brown color
Picric acid test (15)—orange-red color or precipitate

Group II-B-1

	RESORCINOL TEST (16) STRONG ALKALI	RESORCINOL TEST (17) WEAK ALKALI	PHLOROGLUCINOL TEST (18)
Chloral hydrate	Bright red	Green fluorescence	Lilac, changing to orange, to red
Chloroform	Red, orange-red	No fluorescence	Neg.
Carbon tetrachloride	Red, orange-red	No fluorescence	Neg.

Group II-B-2a

	CHROMOTROPIC ACID TEST (19)	FUCHSIN TEST (20)
Acetaldehyde	Neg.	Neg.
Formaldehyde	Pos.	Pos.

Group II-B-2b-1

	OXIDATION (21) FOLLOWED BY CHROMOTROPIC ACID TEST (19)	OXIDATION (21) FOLLOWED BY FUCHSIN TEST (20)
Methyl alcohol	Pos.	Pos.
Ethyl alcohol	Neg.	Neg.

Group II-B-2b-2

	ELECTROLYTIC REDUCTION (22)	REDUCTION DIAZOTIZATION (23)	FORMALDEHYDE PLUMBITE TEST (24)	AMMONIUM MOLYBDATE TEST (25)
Nitrobenzene	Green color	Pos.	Neg.	Neg.
Carbon disulfide	Neg.; yellow color of reagent	Neg.	Yellow, brown, or black precipitate or solution	Blue or blue-green color

TABLE III. DIFFERENTIATING TESTS—ALKALINE STEAM DISTILLATE

	NESSLER'S TEST (26)	p-NITROANILINE TEST (27)	PICRIC ACID TEST (28)	OXIDATION NITROPRUSSIDE TEST (29)	DIAZOTIZATION AND COUPLING (11)	HYPOCHLORITE TEST (10)
Amphetamine	Pos.	Pos.	Amorphous or no precipitate	Neg.	Neg.	Neg.
Nicotine	Neg.	Neg.	Characteristic needle-shaped crystals	red color	Neg.	Neg.
Aniline	Neg.	Pos.	Amorphous or no precipitate	Neg. or black	Pos.	Pos.

Tests for the presence of aniline should be carried out in both the acid and alkaline steam distillates, since this compound is steam volatile at both pH 5.0 and pH 8.0.

Carbon tetrachloride is positive to the tests listed for chloroform, but only in much higher concentrations. Therefore, if these tests are positive, the possibility of the presence of carbon tetrachloride should be kept in mind. However, if the steam distillate is positive to these tests and does not have the odor of carbon tetrachloride, it is likely to contain chloroform.

Acetone is not included in the scheme. Since ketone bodies are normal intermediaries of fat metabolism, steam distillates of homogenates from tissues (such as liver and kidney) regularly give positive reactions if sensitive tests for acetone are performed.

SUMMARY

An analytic scheme for the detection of the more common steam-volatile organic poisons is described.

REFERENCES

1. Landolt, H.: Bromwasser als Reagens auf Phenol und verwandte Körper, Ber. deut. chem. Ges. **4**: 770-773, 1871.
2. (a) Millon, E.: Sur un réactif propre aux composes proteiques, Compt. rend. Acad. Sci. **28**: 40-42, 1849.
   (b) Almén, A.: Die relative Empfindlichkeit der Phenol- und Salicylsaurereaktionen, Ztschr. f. anal. Chem. **17**: 106-109, 1878.
   (c) Plugge, P. C.: Salpetrige Säure haltiges Quecksilbernitrat als Reagens auf aromatische Körper mit einer Gruppe OH am Benzolkern, Arch. d. Pharm. **9**: 228, 1890.
3. Neubauer, C.: Qualitative Ermittelung organischer Körper, Ztschr. f. anal. chem. **14**: 370-384, 1875.
4. Sundberg, T.: Reactions for Hydrocyanic Acid, Svensk Kem. Tids. **33**: 112-113, 1921
5. (a) Ross, J. H.: A Color Test for Chloroform and Chloral Hydrate, J. Biol. Chem. **58** 641-642, 1923.
   (b) Yodomigawa, K.: A New Microcolorimetric Method for Chloroform, Bull. Hokuetsu Med. Soc. **43**: 355-363, 1928.
6. Eegriwe, E.: Reaktionen und Reagenzien zum Nachweis organischer Verbindungen II, Ztschr. f. anal. Chem. **95**: 323-327, 1933.
7. (a) Liebermann, C.: Ueber die Einwirkung der salpetrigen Säure auf Phenole, Ber. deut. chem. Ges. **7**: 247-250, 1874.
   (b) Decker, H., and B. Solonina: Zur Kenntniss der Nitrosophenolfarbstoffe, Ber. deut. chem. Ges. **35**: 3217-3225, 1902.
8. Ware, A. H.: New Specific Tests for Distinguishing Carbolic Acid, the Cresols and Certain Other Phenols, Analyst **52**: 335-337, 1927.
9. (a) Levine, V. E.: Studies in Toxicologic Chemistry. I. The Detection of the Opium Alkaloids by Selenious-Sulfuric Acid: The Specificity of This Reagent for the Phenolic Group, J. Lab. & Clin. Med. **11**: 809-816, 1926.
   (b) Mecke: Ein neues Reagens auf Alkaloide, Z. öffent, Chem. **5**: 351-354, 1899.
10. (a) Jacquemin, M. E.: Acide érythrophénique, réaction nouvelle du phénol et de l'aniline, Compt. rend. Acad. Sci. **76**: 1605-1607, 1873.
    (b) Idem: De la rhodéine, reaction nouvelle de l'aniline, ibid. **83**: 226-227, 1876.
    (c) Dehn, W. M., and S. F. Scott: Some Characteristic Color Reactions Produced by Sodium Hypobromite, J. Am. Chem. Soc. **30**: 1418-1423, 1908.
11. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. **128**: 537-550, 1939.
12. Wasicky, R.: Eine neue sehr empfendliche Farbenreaktion des Atropins, Hyoscyamins und Skopolamins, Ztschr. anal. Chem. **54**: 393-395, 1915.
13. Fulton, C. C.: A Test for Phenols and for Copper, Am. J. Pharm. **105**: 25-29, 1933.
14. Lavialle, P., and Varenne, L.: Characterisation de l'acide hydrocyanique en toxicologie, J. de pharm. et chim. Par. **12**: 74-81, 1915.

15. (a) Braun, C. D.: Einige Reaktionen auf Blausäure, Ztschr. f. anal. Chem. **3**: 463-465, 1864.
(b) Smith, R. G.: Method for the Quantitative Determination of Cyanide in Small Amounts, J. Am. Chem. Soc. **51**: 1171-1174, 1929.
16. Schwarz, C., and Well, H.: Beiträge zur Prüfung und Reinigung der Käuflichen Chloroformsorten, Pharm. Ztg. Berl. **33**: 551-53, 1888.
17. Jaworowski, A.: Einige Notizen über gerichtlich—chemische Untersuchungen, Pharm. Z. f. Russland **33**: 373-74, 1894.
18 (a) Eegriwe, E.: Reaktionen und Reagenzien zum Nachweis organischer Verbindungen IV, Ztschr. f. anal. Chem. **110**: 22-25, 1937.
(b) Feigl, F.: Qualitative Analysis by Spot Tests, ed. 3, New York, 1946, Elsevier Pub. Co.
19. (a) Schiff, H.: Eine neue Reihe organischer Diamine, Ann. **140**: 92, 1866.
(b) von Bitto, B.: Ueber Farbenreaktionen der Aldehyde und Ketone, Ztschr. f. anal. Chem. **36**: 369-376, 1897.
20. Feigl, F., Anger, V., and Frehden, O.: Spot Tests for the Detection of Organic Compounds, IV, Mikrochemie **15**: 181-189, 1934.
21. Hofmann, K. A.: Ueber Eisenpentacyanverbindungen, Ann. **312**: 21, 1900.
22. (a) Feigl, F., and Weisselberg, K.: Beiträge zum Nachweis von Schwefelkohlenstoff, Ztschr. f. anal. Chem. **83**: 93-104, 1931.
(b) Gadamer, J.: Lehrbuch der chemischen Toxikologie, Göttingen, 1909, Vandenhoeck und Ruprecht, p. 286.
23. Deniges, G.: Identification of Benzedrine, Bull. trav. soc. pharm. Bordeaux **83**: 3-6, 1945.
24. (a) Beyer, K. H., and Skinner, J. T.: Detoxication and Excretion of Benzedrine, J. Pharmacol. & Exper. Therap. **68**: 419-432, 1940.
(b) Beyer, K. H.: Color Reactions of Sympathomimetic Amines With Diazonium Compounds, J. Am. Chem. Soc. **64**: 1318-1322, 1942.
25. (a) Wagenaar, M.: Microchemical Reactions for Nicotine, Pharm. Weekbl. 66: 773-776, 1929.
(b) Ionescu-Matui, A., and Iliesco, E.: Contribution à la connaissance de l'identification des alcaloides comme picrates, J. Pharm. Chim. **23**: 117-141, 1936.
(c) Whitmore, W. F., and Wood, C. A.: Chemical Microscopy of Some Toxicologically Important Alkaloids, Mikrochemie **27**: 249-334, 1939.
26. Policarpo, L.: A New Color Reaction for Nicotine, Anales farm. bioquim. (Buenos Aires) **12**: 117-125, 1941.
27. (a) Autenrieth, W.: Die Auffindung der Gifte und stark wirkenden Arzneistoffe, Ann Arbor, 1944, J. Edwards, p. 31.
(b) Bamford, F.: Poisons, Philadelphia, The Blakiston Company, p. 55.
(c) Gadamer, J.: Lehrbuch der chemischen Toxikologie, Göttingen, 1909, Vandenhoeck und Ruprecht, pp. 329, 335.
(d) Lewin, L.: Gifte und Vergiftungen, Berlin, 1929, Georg Stilke, p. 549.

A METHOD FOR THE DETERMINATION OF TITERS BETWEEN 10 AND 100 IN THE QUANTITATIVE COMPLEMENT FIXATION TEST FOR SYPHILIS

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THE value of quantitative tests in diagnosis and control of treatment of syphilis is now widely appreciated. The complement fixation test developed in this laboratory is based on essential quantitative principles[1-9] and in practice yields an accurate and reproducible index of titer.[10-14] Studies have long been directed to a simplification of the method that would not sacrifice the advantages of an optimally adjusted system. The present report describes an abbreviated technique in which four or six dilutions of the specimen are titrated with six 50 per cent units of complement. The final values are read directly from tables. The method is well suited to the testing of sera from patients under treatment for primary or secondary syphilis and, indeed, for all but an occasional specimen.

The rationale and technique for determining titers greater than 10 in the quantitative complement fixation test for syphilis have been described in previous publications.[15, 16a] The procedure[15] first used employed a graphic expression of the results, on the basis of which titers were determined by linear extrapolation. This method required testing several dilutions of a specimen with varying amounts of antigen and 3, 6, 9, and 12 units of complement and was applicable to specimens of all degrees of reactivity. Titers from slightly over 10, that is 11 or 12, to titers of 300 to 500 and even up to 2,000 were obtained. A procedure in which 6 and 9 units of complement were used, each with a single amount of antigen, was later found to simplify the technique considerably without great sacrifice of accuracy.[16a, 17] The graphic method of estimating titers was supplanted by tables of calculated values. Experience with this method in testing sera from patients under treatment for primary and secondary syphilis indicated that titers up to 100 furnish as much information as is required in the management of such cases. The procedure here described was designed primarily to determine titers in the range of 10 to 100. It can nevertheless be extended readily to determine higher titers.

A preliminary titer range test is performed to select the amounts of serum to be tested.

Technique of the Titer Range Test for Use With Serum.—The technique of the titer range test differs slightly from the one previously described.[16e] Proceed as indicated in Table I. Prepare the 1:5 dilution of the reacting serum to be tested, using as a diluent pooled human sera that are clear and colorless, are not anticomplementary, and have failed to react in serologic tests for syphilis. Use

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this diluent also to adjust the volume of serum to 0.05 ml. in tube 2. Inactivate the diluent for thirty minutes each day before use. Controls of the anticomplementary activity of the specimen are included in Table I but are not essential. They are of value in estimating the approximate titer.

TABLE I. PRELIMINARY TITER RANGE TEST OF SERUM

TUBE	REACTING SERUM		EQUIVALENT AMOUNT OF SERUM (ML.)	POOLED NON-REACTING SERA (ML.)	CARDIOLIPIN ANTIGEN #72 DILUTED 1:67 (ML.)	UNITS OF COMPLEMENT PER 0.1 ML. (ML.)		SALT SOLUTION (ML.)	PERIOD OF FIXATION	SENSITIZED SHEEP CELLS (ML.)	PERIOD OF HEMOLYSIS
	DILUTION	AMOUNT PIPETTED (ML.)				1	6				
1	1:5	0.05	0.01		0.1		0.1	0.05	4 hr., 3-6° C.	0.2	15 min. 37° C.
2	1:5	0.02	0.004	0.03	0.1		0.1	0.05		0.2	
3	Undiluted	0.05	0.05			0.1		0.15		0.2	
4	Undiluted	0.05	0.05			0.2		0.05		0.2	

Tubes 3 and 4 can be omitted if an estimated titer is not desired.

The titer is expressed as the ratio of the amount of complement required to give 50 per cent hemolysis with 0.05 ml. of serum (or 0.2 ml. of cerebrospinal fluid) and an optimal dose of antigen to the amount required to give 50 per cent hemolysis with the same amounts of the specimen alone. For convenience, the findings with specimen and antigen are referred to as (S+A) and with the specimen alone, as (S). The titer is the ratio $\frac{(S+A)}{(S)}$, and is expressed in two significant figures. Record the percentage of hemolysis in each tube by comparison with a color standard.[16f] Determine the values of (S+A) by reference to Table II.

TABLE II. VALUES OF (S + A)* FOR TESTS WITH 6 UNITS OF COMPLEMENT, ANTIGEN, AND DILUTED SERUM

PER CENT HEMOLYSIS	SERUM DILUTED TO 1:									
	2.5	3.0	4.0	5.0	6.0	7.5	9.5	12.0	15.0	20.0
	EQUIVALENT ML. OF UNDILUTED SERUM									
	0.02	0.0167	0.0125	0.01	0.0083	0.0067	0.0053	0.004	0.0033	0.0025
5	23	27	36	46	55	68	86	114	137	180
10	21	25	33	42	50	62	79	104	125	170
15	20	23	31	39	47	59	74	98	117	160
20	19	22	30	37	44	56	70	93	111	150
25	18	22	29	36	43	54	68	90	108	140
30	17	20	27	34	41	51	65	85	102	140
35	17	20	27	34	40	50	64	84	101	130
40	16	19	26	32	38	48	61	80	96	130
45	16	19	25	31	37	47	59	78	93	120
50	15	18	24	30	36	45	57	75	90	120
55	15	17	23	29	35	44	55	73	87	120
60	14	17	22	28	34	42	53	70	84	110
65	14	16	22	27	32	41	51	68	81	110
70	13	16	21	26	31	39	49	65	78	100
75	12	15	20	25	29	37	47	61	74	98
80	12	14	19	24	28	35	45	59	71	94
85	11	13	18	22	26	33	42	55	66	88
90	10	12	16	20	24	30	38	50	60	80

*(S + A). The units of complement required for 50 per cent hemolysis with specimen and antigen (Table IX, line 4) multiplied by the dilution factor.

Interpretation of the Titer Range Test With Serum.—When complete hemolysis is obtained with both amounts of serum tested, the value for (S+A) is less than 20, and amounts from 0.02 through 0.01 ml. should be tested to determine the exact titer (see Table II). Five hundredths of a milliliter of serum diluted from 1:2.5 through 1:5 are used in the test.

When partial hemolysis is obtained with either amount in the titer range test, determine from Table II the amounts of serum and the sequence of dilutions to use; for example, if 10 per cent hemolysis occurs with 0.01 ml. and complete hemolysis with 0.004 ml., a value between 42 and 50 is indicated. Therefore, test the serum diluted from 1:4 through 1:12.5. These dilutions include one amount above and one below the range of values indicated for (S+A) to allow for possible variations in complement.

When no hemolysis is obtained in either amount in the titer range test, the value of (S+A) is greater than 100. If the exact titer is desired in such instances, titrate amounts smaller than 0.004 milliliter. For this purpose, use a series of dilutions ten times as great as those given in Table II.

Table III indicates the amounts of reacting and nonreacting serum used to prepare the dilutions.

TABLE III. AMOUNTS OF REACTING AND NONREACTING SERUM USED IN THE PREPARATION OF DILUTIONS

DILUTION	REACTING SERUM (ML.)	NONREACTING SERUM (ML.)	REACTING SERUM CONTAINED IN 0.05 ML. OF EACH DILUTION (ML.)	REMARKS
1:2.5	0.08	0.12	0.02	Higher dilutions in the same progression can be prepared for determining titers greater than 100. Do not pipette less than 0.02 ml. of reacting serum to prepare dilutions for these tests.
1:3	0.07	0.14	0.0167	
1:4	0.05	0.15	0.0125	
1:5	0.05	0.2	0.01	
1:6	0.04	0.2	0.0083	
1:7.5	0.04	0.26	0.0067	
1:9.5	0.02	0.17	0.0053	
1:12.5	0.02	0.23	0.004	
1:15	0.02	0.28	0.0033	
1:20	0.02	0.38	0.0025	

Technique of the Titer Range Test for Use With Cerebrospinal Fluid.—When the titer of the cerebrospinal fluid is greater than 10, perform a titer range test as outlined in Table IV. Use Table V for determining the value of (S+A). Depending upon the range of reaction indicated by this test, use, in the final titration, varying amounts of a 1:2 dilution made with salt solution in the sequence of amounts given in Table V. If titers greater than 100 are desired, use a 1:20 dilution.

Technique for Determination of Titers Between 10 and 100 for Both Serum and Cerebrospinal Fluid.—The method of testing is illustrated in Tables VI and VII, and typical results are shown in Table VIII. The calculated values for (S+A) corresponding to the degrees of hemolysis obtained in the test of the different amounts of the specimen used are shown in Tables II and V. These

TABLE IV. PRELIMINARY TITER RANGE TEST OF CEREBROSPINAL FLUID

TUBE	UNDILUTED CEREBROSPINAL FLUID (ML.)	SALT SOLUTION (ML.)	CARDIOLIPIN ANTIGEN #72 DILUTED 1:90 (ML.)	UNITS OF COMPLEMENT PER 0.1 ML. (ML.)		PERIOD OF FIXATION	SENSITIZED SHEEP CELLS (ML.)	PERIOD OF HEMOLYSIS
				1	6			
1	0.04	0.16	0.1		0.1	4 hr.	0.2	15 min.,
2	0.02	0.18	0.1		0.1	3-6° C.	0.2	37° C.
3	0.2	0.1		0.1			0.2	
4	0.2			0.2			0.2	

Tubes 3 and 4 can be omitted if an estimated titer is not desired.

TABLE V. VALUES FOR (S + A)* IN TESTS WITH ANTIGEN, 6 UNITS OF COMPLEMENT, AND CEREBROSPINAL FLUID IN THE GIVEN AMOUNTS

PER CENT HEMOLYSIS	AMOUNTS OF CEREBROSPINAL FLUID DILUTED TO 1:2									
	0.16	0.13	0.1	0.08	0.07	0.06	0.05	0.04	0.03	0.02
	EQUIVALENT AMOUNTS OF UNDILUTED CEREBROSPINAL FLUID									
	0.08	0.065	0.05	0.04	0.035	0.03	0.025	0.02	0.015	0.01
5	23	28	36	46	52	61	73	91	121	180
10	21	25	33	42	47	55	66	83	110	170
15	20	24	31	39	45	52	62	78	104	160
20	19	23	30	37	42	49	59	74	98	150
25	18	22	29	36	41	48	58	72	96	140
30	17	21	27	34	39	45	54	68	90	140
35	17	21	27	34	38	45	54	67	89	130
40	16	20	26	32	37	43	51	64	85	130
45	16	19	25	31	35	41	50	62	82	120
50	15	18	24	30	34	40	48	60	80	120
55	15	18	23	29	33	39	46	58	77	120
60	14	17	22	28	32	37	45	56	74	110
65	14	17	22	27	31	36	43	54	72	110
70	13	16	21	26	30	35	42	52	69	100
75	12	15	20	25	28	33	39	49	65	100
80	12	14	19	24	27	31	38	47	63	94
85	11	14	18	22	25	29	35	44	59	88
90	10	12	16	20	23	27	32	40	53	80

*(S + A). The units of complement required for 50 per cent hemolysis with specimen and antigen (Table IX, line 4) multiplied by the dilution factor.

TABLE VI. DETERMINATION OF TITERS OF SERUM UP TO 100

DILUTION OF SPECIMEN	AMOUNT PIPETTED (ML.)	CARDIOLIPIN ANTIGEN #72 DILUTED 1:67 (ML.)	UNITS OF COMPLEMENT PER 0.1 ML. (ML.)		SALT SOLUTION (ML.)	PERIOD OF FIXATION	SENSITIZED CELLS (ML.)	PERIOD OF HEMOLYSIS
			1	6				
Undiluted	0.05		0.1		0.15	4 hr.,	0.2	15 min.,
Undiluted	0.05		0.1		0.15	3-6° C.	0.2	37° C.
Undiluted	0.05		0.2		0.05		0.2	
1st dilution	0.05	0.1		0.1	0.05		0.2	
2nd dilution	0.05	0.1		0.1	0.05		0.2	
3rd dilution	0.05	0.1		0.1	0.05		0.2	
4th dilution	0.05	0.1		0.1	0.05		0.2	
5th dilution	0.05	0.1		0.1	0.05		0.2	
6th dilution	0.05	0.1		0.1	0.05		0.2	

values were obtained by multiplying the units of complement required for 50 per cent hemolysis in the presence of the diluted specimen and the maximally reacting dose of antigen, as read from line 4 of Table IX, by the dilution factor;

that is, 0.05 ml. divided by the milliliters of serum used. For example: in a test in which 65 per cent hemolysis occurs with 0.05 ml. of a 1:5 dilution of serum, (S+A) would be $5.4 \times \frac{0.05}{0.01}$, or 27. Similarly, in a test of 0.02 ml. of cerebrospinal fluid, 40 per cent hemolysis would correspond to an (S+A) value of $6.4 \times \frac{0.2}{0.02}$, or 64.

TABLE VII. DETERMINATION OF TITERS OF CEREBROSPINAL FLUID UP TO 100

DILUTION OF SPECIMEN	AMOUNT PIPETTED* (ML.)	SALT SOLUTION† (ML.)	CARDIOLIPIN ANTIGEN #72 DILUTED 1:90 (ML.)	UNITS OF COMPLEMENT PER 0.1 ML. (ML.) 1	3	6	PERIOD OF FIXATION	SENSITIZED CELLS (ML.)	PERIOD OF HEMOLYSIS
Undiluted	0.2	0.1		0.1			4 hr., 3-6° C.	0.2	15 min., 37° C.
Undiluted	0.2			0.2				0.2	
Undiluted	0.2	0.1			0.1			0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	

*Varies according to range of activity (see Table V).
†Balance to a volume of 0.4 ml. according to amount of dilution pipetted.

TABLE VIII. TYPICAL RESULTS OF TESTS TO DETERMINE THE TITER OF SERUM OR CEREBROSPINAL FLUID

SPECIMEN	DILUTION OF SPECIMEN	AMOUNT PIPETTED (ML.)	AMOUNT TESTED (ML.)	PER CENT HEMOLYSIS WITH UNITS OF COMPLEMENT 1	2	3	6	VALUE OF (S)* (READ FROM TABLE IX)	VALUE OF (S+A)† (READ FROM TABLE II OR V)	TITER (S+A)† / (S)*
Serum	Undiluted	0.05	0.05	25				1.23		
	Undiluted	0.05	0.05	25				1.23		
	Undiluted	0.05	0.05		95					
	1:4	0.05	0.0125				0		>36	
	1:5	0.05	0.01				15		39	
	1:6	0.05	0.0083				30		41	
	1:7.5	0.05	0.0067				55		*44*	*44*=36 / 1.23
	1:9.5	0.05	0.0053				85		42	
	1:12.5	0.05	0.004				100		<50	
Cerebrospinal fluid	Undiluted	0.2	0.2	45				1.04		
	Undiluted	0.2	0.2		100					
	Undiluted	0.2	0.2			100				
	1:2	0.16	0.08				0		>23	
	1:2	0.13	0.065				5		28	
	1:2	0.1	0.05				25		*29*	*29*=28 / 1.04
	1:2	0.08	0.04				60		28	
	1:2	0.07	0.035				80		27	
	1:2	0.06	0.03				90		27	

*(S), The reaction with the specimen alone.
†(S + A), The reaction with specimen and antigen.

TABLE IX. UNITS OF COMPLEMENT REQUIRED FOR 50 PER CENT HEMOLYSIS INDICATED BY DEGREES OF HEMOLYSIS OBTAINED WITH DIFFERENT AMOUNTS OF COMPLEMENT IN TESTS WITH SPECIMENS (SERUM OR CEREBROSPINAL FLUID) OR WITH SPECIMENS AND ANTIGEN

UNITS OF COMPLEMENT	PER CENT HEMOLYSIS																		
	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
1	1.78	1.54	1.39	1.32	1.23	1.19	1.12	1.09	1.04	1.0	0.96	0.93	0.88	0.85	0.81	0.76	0.71	0.65
2	3.51	3.06	2.81	2.63	2.49	2.37	2.26	2.16	2.07	2.0	1.92	1.84	1.76	1.69	1.6	1.52	1.42	1.29
3	5.0	4.5	4.1	3.9	3.7	3.5	3.4	3.2	3.1	3.0	2.9	2.8	2.6	2.5	2.4	2.3	2.1	1.9
6	9.1	8.3	7.8	7.4	7.2	6.8	6.7	6.4	6.2	6.0	5.8	5.6	5.4	5.2	4.9	4.7	4.4	4.0
12	15.0	14.4	14.0	13.5	13.3	13.0	12.7	12.5	12.2	12.0	11.7	11.5	11.2	10.8	10.5	10.1	9.5	8.8

The 50 per cent unit values of the reactions in the controls of the specimen without antigen are also read from Table IX. Determine the titer by dividing the maximum value for (S+A), Table II or V, by the maximum value for (S), Table IX. Consider specimens anticomplementary if the reaction without antigen is 2.0 or greater. Anticomplementary reactions can be measured, but since it is doubtful how much this property of serum or cerebrospinal fluid affects the fixation due to syphilitic reagin, the titers obtained with anticomplementary specimens must be considered approximate values only. Always include controls (S) with sufficient complement to produce partial degrees of hemolysis in tests of anticomplementary specimens. Whenever the value of (S) is less than 1, the value of (S+A) represents the titer of the specimen; that is, a value of less than 1 is never used in the denominator of the fraction $\frac{(S+A)}{(S)}$.

Evaluation of Results.—Compare the maximum reaction obtained with serum and antigen in the final titration with that obtained in the titer range

TABLE X. CHART TO AID IN DETERMINING RELATIVE DISCREPANCY (IF B IS LESS THAN INDICATED, THE RELATIVE DISCREPANCY BETWEEN THE NUMBERS (A AND B) IS LESS THAN 25 PER CENT)

A	B	A	B	A	B	A	B
98	126	182	234	336	432	616	792
101.5	130.5	185.5	238.5	343	441	630	810
105	135	189	243	350	450	644	828
108.5	139.5	192.5	247.5	357	459	658	846
112	144	196	252	364	468	672	864
115.5	148.5	203	261	371	477	686	882
119	153	210	270	378	486	700	900
122.5	157.5	217	279	385	495	714	918
126	162	224	288	392	504	728	936
129.5	166.5	231	297	406	522	742	954
133	171	238	306	420	540	756	972
136.5	175.5	245	315	434	558	770	990
140	180	252	324	448	576	784	1008
143.5	184.5	259	333	462	594	812	1044
147	189	266	342	476	612	840	1080
150.5	193.5	273	351	490	630	868	1116
154	198	280	360	504	648	896	1152
157.5	202.5	287	369	518	666	924	1188
161	207	294	378	532	684	952	1224
164.5	211.5	301	387	546	702	980	1260
168	216	308	396	560	720		
171.5	220.5	315	405	574	738		
175	225	322	414	588	756		
178.5	229.5	329	423	602	774		

test. Determine the relative discrepancy either by dividing the difference of the two results by the mean or by comparing them with the pairs of numbers listed in Table X. Repeat the examination of specimens if the findings show more than 25 per cent relative discrepancy. If controls without antigen have been included in the titer range test, comparison of the ratios, $\frac{(S+A)}{(S)}$, rather than of the values, (S+A), usually shows closer agreement, and less repetition is required.

DISCUSSION

In routine practice in this laboratory about 85 per cent of specimens received for serologic tests for syphilis can be reported as not reacting on the basis of two preliminary tests—one of complement fixation[16b] and the other of precipitation[16c]; both tests are oversensitive and require one tube each. The remaining reacting sera are tested in the six-tube quantitative procedure,[16d] in which titers up to 10 are determined. Specimens having titers greater than 10 —about half of those examined quantitatively—are then tested in a two-tube titer range test, which indicates the dilutions to be used in determining the exact titer. The successive tests reduce to a minimum the number of specimens requiring titration in dilution and some advantage is derived from the confirmatory evidence they afford.

Studies are being continued to modify the technique of the six-tube quantitative test to extend the range of determinable titer somewhat beyond 10. It is hoped that this will largely eliminate, or permit better interpretation of, the so-called atypical reaction in these lower ranges and also avoid the inaccurate index of titer occasionally encountered in the tube containing 12 units of complement which now requires a supplementary test to correct. Possibly a greater flexibility of procedure will be provided also.

SUMMARY

A simplified technique for determining titers between 10 and 100 in the quantitative complement fixation test for syphilis is described. Two amounts of serum or cerebrospinal fluid are tested to determine the range of reactivity, and from four to six dilutions are tested to determine the exact titer. Although the method is designed primarily for determination of titers under 100, it is also applicable to determinations of higher titers.

The procedure results in material saving of time and reagents as compared with previous methods.

REFERENCES

1. Wadsworth, A., Maltaner, Frank, and Maltaner, Elizabeth: The Quantitative Determination of the Fixation of Complement by Immune Serum and Antigen, J. Immunol. **21**: 313-340, 1931.
2. Maltaner, E. J., and Maltaner, Frank: A Further Note on the Quantitative Determination of the Fixation of Complement by Immune Serum and Antigen, J. Immunol. **29**: 151-155, 1935.
3. Wadsworth, A., Maltaner, Frank, and Maltaner, Elizabeth: Quantitative Studies of the Reaction of Complement Fixation With Tuberculous Immune Serum and Antigen, J. Immunol. **35**: 93-103, 1938.
4. Wadsworth, A., Maltaner, Frank, and Maltaner, Elizabeth: Quantitative Studies of the Reaction of Complement Fixation With Syphilitic Serum and Tissue Extract, J. Immunol. **35**: 105-115, 1938.
5. Wadsworth, A., Maltaner, Frank, and Maltaner, Elizabeth: Quantitative Studies of the Complement-Fixation Reaction With Syphilitic Serum and Tissue Extracts: Technic of the Practical Quantitative Test, J. Immunol. **35**: 217-234, 1938.
6. Thompson, W. R., and Maltaner, F.: On the Construction of Graphs and Tables for Evaluation of the Quantitative Complement-Fixation Reactions and Reaction Ratios, J. Immunol. **38**: 147-157, 1940.
7. Rice, C. E.: A Study of the Reliability of Complement Fixation as a Method of Measuring the Activities of Sera of High, Medium, and Low Antibody Titer, J. Immunol. **55**: 1-13, 1947.

8. Kent, J. F., Bukantz, S. C., and Rein, C. R.: Studies in Complement Fixation. I. Spectrophotometric Titration of Complement; Construction of Graphs for Direct Determination of the 50 Per Cent Hemolytic Unit, J. Immunol. 53: 37-50, 1946.
9. Mayer, M. M., Osler, A. G., Bier, Otto G., and Heidelberger, M.: Quantitative Studies of Complement Fixation, Proc. Soc. Exper. Biol. & Med. 65: 66-68, 1947.
10. Thomas, E. W.: The Clinical Significance of Quantitative Serologic Tests for Syphilis, Am. J. Syph. 30: 317-329, 1946.
11. Dattner, B.: Management of Neurosyphilis; Its Diagnosis, Clinical Aspects, and Treatment, N. Y. State Assoc. Pub. Health Laboratories Proc. 26: No. 2, 85-89, 1946.
12. Maillard, E. R., and Orzel, A.: The Value of the Quantitatively Standardized Complement-Fixation Test in the Diagnosis and Treatment of Early Syphilis, Am. J. Syph. 30: 490-494, 1946.
13. Maillard, E. R., and Orzel, A.: The Value of Quantitatively Standardized Laboratory Tests in Neurosyphilis, Am. J. Syph. 31: 506-517, 1947.
14. Maillard, E. R.: Quantitatively Standardized Technics in the Examination of Blood and Cerebrospinal Fluid in Neurosyphilis, N. Y. State Assoc. Pub. Health Laboratories Proc. 26: No. 2, 82-85, 1946.
15. Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, ed. 2, Baltimore, 1939, Williams & Wilkins Company, p. 260.
16. Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, ed. 3, Baltimore, 1947, Williams & Wilkins Company, (a) p. 438-449; (b) p. 452; (c) p. 462; (d) p. 422; (e) p. 439; (f) p. 374.
17. New York (State) Department of Health, Division of Laboratories and Research, Annual Report, Albany, N. Y., 1944, p. 18.

THE PREPARATION OF FROZEN SECTIONS FOR USE IN THE CHEMOSURGICAL TECHNIQUE FOR THE MICROSCOPICALLY CONTROLLED EXCISION OF CANCER

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THE microscopic control of excision which characterizes the chemosurgical technique for the treatment of cancer is attained by the use of frozen sections of specimens removed from the areas under suspicion. As previously described,[1-6] the chemosurgical technique consists of the following steps: (1) the fixation in situ of the tissues by the application of a paste containing a fixative chemical such as zinc chloride; (2) the excision of a layer of fixed tissue which is divided into convenient specimens, the locations of which are mapped on paper and on the lesion; (3) the preparation of frozen sections by cutting through the under surface of each specimen; (4) the scanning of the sections under the microscope so that the remaining areas of cancer can be indicated on the map; (5) reapplication of the fixative to the cancerous parts of the lesion; (6) repetition of the process until the entire area is free of cancer.

This article concerns the microtechnical procedures by which the frozen sections are made. Special consideration of the frozen section technique as applied to the chemosurgical specimen is desirable because of the importance of obtaining complete sections of the specimens which often are rather large and sometimes are friable. The sections must be complete because if any part of a specimen is lost during the sectioning process, the operator cannot know whether the lost tissue was cancerous or noncancerous and therefore cannot know whether further treatment is required. These considerations have necessitated the development of some new details of technique. The usual procedures for the preparation of frozen sections along with the modifications which have been found useful are given herewith.

TECHNIQUE

Mounting of the Specimens.—The specimens as they are excised from the lesion are usually flat pieces of tissue which are approximately 1 cm. across. Since it is the under surface of these specimens that is to be investigated, the specimen is frozen on the stage by placing it in a small pool of albumin solution* which has been partially frozen by rapidly opening and closing the valve

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*Albumin solution is used because when it freezes, the ice is not so hard and brittle as when plain water is used. It also holds the specimen on the stage more firmly. It is made by adding approximately 0.5 Gm. of albumin to 30 c.c. of water and preserving with a drop of 95 per cent phenol.

several times. Although the specimen has ordinarily been flattened before placement upon the stage, it is often necessary to elevate one or more edges by cutting into the ice under the specimen with a knife. If the tissue is irregular in thickness it may be necessary to flatten it with the broad surface of a scalpel handle before mounting it on the stage.

It is important that the specimen be oriented so that the microtome knife strikes the narrowest part of the specimen first and moves diagonally over the greatest area (Fig. 1, *A*, *B*, *C*, and *D*). Specimens containing connective tissue, muscle fibers, or cartilage should be mounted so that the fibers (or the long axis of the cartilage) are parallel to the direction of movement of the knife

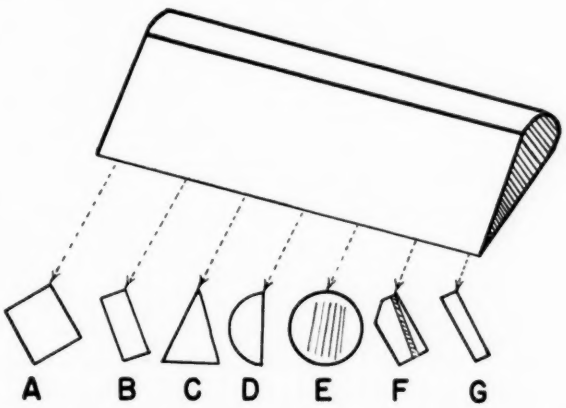

Fig. 1.—Diagrams showing the recommended angle at which the microtome knife should meet specimens of different shapes and consistencies.

(Fig. 1, *E* and *F*). If a vertical section of the flat piece of tissue is cut, its long axis is placed at an angle of about 65° in relation to the knife (Fig. 1, *G*). The specimen should never be placed so that the entire edge meets the knife at the same time because this will crush the tissue, cause the knife to jump over the specimen, or push the entire block off the stage.

Specimens containing skin are placed so that the skin edge is not cut first. Likewise, tissues which are heterogeneous in consistency are placed so that the softer part of the tissue is cut first. This provides a firm backing for the knife to cut against.

Cutting of the Specimen.—When the block of ice has been frozen to the optimum degree of hardness, the section is cut with the microtome knife which is usually used dry and at room temperature. Each section that is cut adheres to the blade and is removed with the moist finger and placed in a glass dish of cold water. If the tissues are fragile this operation must be done gently. If the tissues are pulled apart by the surface tension as they are placed in the water, it is advisable to add a few drops of an aqueous solution of a surface tension depressant such as Zephiran.

Sections are best cut at a thickness of 15 to 25 microns. If the tissues are cut thinner, those that do not contain fairly large amounts of connective

tissue will fall apart. Cancer tissues with a meager connective tissue stroma, fatty tissues, and tissues heavily infiltrated with leucocytes are likely to be fragile; while tissue containing cartilage and considerable amounts of connective tissue will hold together even though cut very thin.

It is particularly important that the knife be kept very sharp. Ordinarily it should be stropped daily and honed weekly. For best results the microtome should be one which is constructed so that the knife is held firmly. A knife-holder which slides on tracks, such as in the Bausch and Lomb clinical microtome, is satisfactory from this standpoint.

Ordinarily, four or five complete sections are cut from each specimen. The water in the dish is changed after cutting each specimen so as to avoid mixing of the sections from different specimens.

Mounting of the Sections.—The sections are worked onto the slide with a curved metal teasing needle. If there is a tendency for the specimen to break up, it is important that the fragments be pieced together so that no part of the section is missing. The edges of the specimens usually have been marked with coloring materials (merbromin, washing bluing, and India ink) for the purpose of orientation when the sections are examined under the microscope. These markings which persist throughout the staining process aid in the rearrangement of any fragments which may fall out. Any folded areas may be remounted by floating off that area and remounting without detaching the whole section. If the section is curled up it may be uncurled by attaching one edge to the slide and drawing the slide out of the water so as to unroll it. If the section is not too fragile, it frequently is possible to work the roll loose by gently moving it around in the water with the wire teaser.

It usually is advisable to put three sections on one slide, particularly if they are fragile and if there is some likelihood of the sections being incomplete. However, one good section is all that is necessary. The slide is then blotted carefully and thoroughly with a very dry soft towel, preferably an old one that has the lint washed from it. It is best to blot only one of the sections first to see if the section will cling to the towel. If it does so, the other sections may be fixed to the slide by passing it through an alcohol flame several times. The slide is numbered with a wax pencil and put in 95 per cent alcohol to await staining.

Staining.—The various solutions used in the staining process are kept in Coplin jars. These jars contain five slots so it is convenient to run five slides at a time, keeping slide number one in slot number one, slide number two in slot number two, and so forth. Some of the slides may be run ahead as long as they are kept in their proper slot so that there is no danger of getting the sections mixed.

The procedure for the dehydration, staining, clearing, and mounting of the sections is as follows.

1. *Ninety-five per cent alcohol:* The slide may be left in this solution for any time over fifteen seconds.

2. *Cellulose nitrate (Parlodion*) solution:* The slide is quickly dipped

*Mallinckrodt Chemical Works, St. Louis, Mo.

in this solution which should be rather thin. If it is too thick, it and the sections tend to peel off the slide. Moreover, a thick layer prevents the ready penetration of the stain and prolongs the process. The solution is made by dissolving one stick (1 Gm.) of parlodion in 30 c.c. of a solution of equal parts of ether and 95 per cent alcohol. After dipping in the cellulose nitrate solution, the slide is dried by immediately blotting firmly with a clean dry towel.

3. *Hematoxylin:* Approximately two minutes is the usual staining time but the slide may be left in longer if the tissue does not take the stain well. Delafield's hematoxylin[7] is used. This stain lasts indefinitely but it is advisable to filter off the precipitate every day before using. After staining, the excess dye is washed off the slide in a dish of water.

4. *Acid alcohol:* This solution is just strong enough to destain the average section with one dip. It is made by adding 25 c.c. of concentrated hydrochloric acid to 500 c.c. of 70 per cent ethanol. This solution is changed once a week or more often if necessary.

5. *Alkaline water:* If the tap water is alkaline, it is convenient to let the slide stand in a dish of water to alkalize. Otherwise a weak solution of sodium bicarbonate is used and the sections are allowed to remain in the solution until they are blue. This usually takes one or two minutes. Only one slide should be in the solution at a time because the sections may loosen and come off at this time and be mixed. If a section floats off it may be mounted as before, blotted with a dry towel, and continued in the staining process. It is advisable to blot the sections at this stage to insure the removal of water and bubbles from under the sections.

6. *Eosin:* The solution is adjusted so that one dip of the section gives adequate staining. The stock solution is made as follows. Dissolve 2.5 Gm. aqueous eosin in 500 c.c. distilled water. Add 4 c.c. of concentrated hydrochloric acid and 1 c.c. of glacial acetic acid. Pour off the supernatant and save the precipitate which is washed six times with water. Filter and dry the precipitate. There will be about 0.5 Gm. of precipitate; this is dissolved in 100 c.c. of 95 per cent alcohol. The stain may be diluted with 95 per cent alcohol if it stains too deeply. Alcohol is also added as needed to replace that lost by evaporation. The solution should be filtered whenever particulate matter gathers in it.

7. *Alcohol and ether:* Treat with this solution for two minutes or more. This solution is made up of equal parts of ether and 95 per cent alcohol and it should be changed daily.

8. *Absolute alcohol:* Treat with this solution for one to two minutes or until all of the parlodion is removed. This solution should be changed once a week or more often if necessary for complete dehydration of the sections.

9. *Carbol-xylol:* Treat with this solution until the tissues are clear. Two minutes or more may be required. The stock solution is made by adding 300 c.c. of xylol to 100 Gm. of phenol crystals. The solution ordinarily is changed once a week.

10. *Xylol:* Treat with this for two minutes or more. Change the solution weekly.

11. *Mount in Clarite* and cover with a 22 by 50 mm. cover slip. This large size cover slip is desirable because several sections are placed on each slide. The clarite solution is made by dissolving 60 Gm. of clarite "X" hydrocarbon resin in 40 c.c. of toluene. Clarite is preferable to balsam because it hardens more rapidly and because it does not become yellow with age.

Sections made with this technique have not deteriorated after periods of over twelve years.

SUMMARY

The microscopic control of excision attained by the chemosurgical treatment of cancer is dependent upon the completeness of the frozen sections of the tissues which have been fixed in situ prior to excision. To make possible these complete sections a number of modifications of the usual frozen section technique have been devised. A procedure embodying these modifications is described. The sections, which are stained with hematoxylin and eosin, are permanent.

REFERENCES

1. Mohs, F. E., and Guyer, M. F.: Pre-excisional Fixation of Tissues in the Treatment of Cancer in Rats, Cancer Research **1**: 49-51, 1941.
2. Mohs, F. E.: Chemosurgery: A Microscopically Controlled Method of Cancer Excision, Arch. Surg. **42**: 279-295, 1941.
3. Mohs, F. E.: Chemosurgical Treatment of Cancer of the Lip: A Microscopically Controlled Method of Excision, Arch. Surg. **48**: 478-488, 1944.
4. Mohs, F. E.: Chemosurgical Treatment of Cancer of the Nose: A Microscopically Controlled Method, Arch. Surg. **53**: 327-344, 1946.
5. Mohs, F. E.: Chemosurgical Treatment of Cancer of the Ear: A Microscopically Controlled Method of Excision, Surgery **21**: 605-622, 1947.
6. Mohs, F. E.: Chemosurgical Treatment of Cancer of the Face: A Microscopically Controlled Method of Excision, Arch. Dermat. & Syph. **56**: 143-156, 1947.
7. Guyer, M. F.: Animal Micrology, ed. 3, Chicago, 1930, University of Chicago Press, p. 9.

Erratum

In the paper by Dominguez, Corcoran, and Page, Mannitol: Kinetics of Distribution, Excretion, and Utilization in Human Beings, which appeared in the October issue of the JOURNAL (**32**: 1192, 1947) equation 13 of Table I should be

$$V_2 = \frac{A_2\ (\beta^2 a + \alpha^2 b)}{\alpha\beta\ (\beta a + \alpha b)}$$

or, in terms of G,

$$V_2 = \frac{G\ (\beta^2 a + \alpha^2 b)}{(\beta a + \alpha b)^2}$$